ACQUIRED TOLERANCE OF HEPATOCELLULAR CARCINOMA CELLS TO SELENIUM DEFICIENCY: A SELECTIVE SURVIVAL MECHANISM

A THESIS SUBMITTED TO

THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

AND THE INSTITUTE OF ENGINEERING AND SCIENCE OF

BİLKENT UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

MELİHA BURCU IRMAK

SEPTEMBER, 2003

TO MY MOTHER, FATHER and

SISTER.....

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope, and in quality, as a thesis for the degree of Doctor of Philosophy.

Assist. Prof. Rengül Çetin Atalay

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope, and in quality, as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Mehmet Öztürk

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope, and in quality, as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Aslıhan Tolun

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope, and in quality, as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Meral Özgüç

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope, and in quality, as a thesis for the degree of Doctor of Philosophy.

Assist. Prof. M. Cengiz Yakıcıer

Approved for the Institute of Engineering and Science

Prof. Dr. Mehmet Baray
Director of the Institute of Engineering and Science

ABSTRACT

Acquired Tolerance of Hepatocellular Carcinoma

Cells to Selenium-Deficiency:

A Selective Survival Mechanism

Meliha Burcu Irmak

Ph.D. in Molecular Biology and Genetics
Supervisor: Assist. Prof. Rengül Çetin Atalay
September, 2003

Selenium-deficiency causes liver necrosis. Selenium is protective against viral hepatitis and hepatocellular carcinoma (HCC). The underlying molecular mechanisms of selenium effects are ill-known. In this study *in vitro* response of hepatocellular carcinoma-derived cell lines to selenium-deficiency is examined alone or in conjunction with Vitamin E and Copper/Zinc. Here we show that *in vitro* selenium-deficiency in a subset HCC-derived 'hepatocyte-like' cell lines causes oxidative stress and apoptosis. The oxidative stress and consequent cell death induced by selenium-deficiency on these cells are reverted by the antioxidant effect of Vitamin E. However, ten among thirteen HCC cell lines are tolerant to selenium-deficiency and escape its deadly consequences. Nine of ten tolerant cell lines have integrated hepatitis B Virus (HBV) DNA in their genomes, and some display p53-249 mutation, indicating past exposure to HBV or aflatoxins, established factors for oxidative stress and cancer risk. Thus, as demonstrated by the gain of survival capacity of apoptosis sensitive cell lines with Vitamin E, such malignant cells have acquired a selective survival advantage that is prominent under selenium-deficient and oxidative stress conditions.

ÖZET

Karaciğer Kanser Hücrelerinin Selenyum Eksikliğine Karşı Edinilmiş Töleransı: Seçici Bir Yaşam Mekanizması

Meliha Burcu Irmak

Doktora Tezi, Moleküler Biyoloji ve Genetik Bölümü
Tez yöneticisi: Yard. Doç. Rengül Çetin-Atalay
Eylül, 2003

Selenyum eksikliği karaciğer nekrozuna sebep olmaktadır ve selenyum viral hepatit ve karaciğer kanserine karşı koruyucu etki gösterir. Selenyum etkilerinin altında yatan moleküler mekanizmalar halen açıklanamamıştır. Bu çalışmada karaciğer kanseri kökenli hücre hatlarının selenyum eksikliğine *in vitro* tepkisi yalnız ya da Vitamin E ve bakır/çinkonun varlığında araştırılmıştır. Araştırmamızda *in vitro* selenyum eksikliğinin bir grup karaciğer kanseri kökenli 'hepatosit-benzeri' hücre hattında oksidatif stres ve apoptoza yol açtığını gösterdik. Bu hücrelerde selenyum eksikliğinin yarattığı oksidatif stres ve buna bağlı hücre ölümü, Vitamin E'nin antioksidan etkisiyle ortadan kalkmaktadır. Ancak onüç karaciğer kanser hücre hattından on tanesi selenyum eksikliğinin ölümcül sonuçlarına karşı tolerans göstermektedir. Toleranslı on hücre hattından dokuz tanesi genomlarında hepatit B virüsü (HBV) DNA'sı bulundurmakta ve de bazıları p53-249 mutasyonu sergilemektedir. Bu da geçmişte bu hücre hatlarının, oksidatif strese ve kansere yol açtığı belirlenen, HBV veya aflatoksinlere maruz kaldıklarının göstergesidir. Sonuç olarak, bu tür malin hücrelerin selenyum eksikliği ve oksidatif stres koşullarında ortaya çıkan edinilmiş seçkin yaşama avantajları, apoptoza duyarlı hücre hatlarının Vitamin E ile yaşama kapasitelerini kazanmalarıyla da gösterilmiştir.

ACKNOWLEDGMENTS

I am thankful to Dr. Rengül Çetin-Atalay for her advices, helps, supports, friendship and contributions to my scientific background throughout my Ph.D. studies.

It is my pleasure to express my special thanks to Prof. Dr. Mehmet Öztürk for his scientific guidance and sharing his invaluable knowledge and experience when we needed. I will always be proud of having had a chance to work with him all my life.

I wish to express my appreciation to Dr. M. Cengiz Yakıcıer for all his supports, helps, understanding, friendship and warm attentions with an everlasting interest during my studies.

Special thanks to my friends….

Yeliz, Ceren, Bala, Hilal and Sevgi for their friendships and helps with the experiments.

Nuri for his helps in the laboratory related to the experiments and the computers and Mehdi for all his helps and supports during my studies.

I am thankful to all MBG family members, the technicians, secretaries and my friends. As the circumstances we shared together made us come closer. Thank you for all your efforts during the generation of this thesis.

My family… Source of my energy… Without your endless support and love, this thesis would not have reached to its aim. Thank you….

Finally, I want to express my gratitude to Prof. Dr. İhsan Doğramacı for giving me the opportunity to complete my Ph.D studies at Bilkent University in Molecular Biology and Genetics Department.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABREVIATIONS

A	Amper
ATP	Adenosin Triphosphate
$CaCl_2$	Calcium chloride
CO_2	Carbondioxide
cont’	Continued
dATP	Deoxyadenosine Triphosphate
ddH_2O	Double distilled water
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
dUTP	Deoxyurasil Triphosphate
EDTA	Ethylenediamine-Tetra-Acetic Acid
EtOH	Ethanol
g	Gram
Gpx-/-	Gpx knockout
h	Hour
lt	Liter
M	Molar
mg	Milligram
mg/ml	Milligram per milliliter
mg/ml	Milligram per millimeter
$MgCl_2$	Magnesium Chloride
ml	Milliliter
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium hydroxide
ng	Nanogram
nM	Nanomolar
nM	Nanometer
PCR	Polymerase Chain Reaction

pmol	Picomole
RNA	Ribonucleic Acid
rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane
Tris	2-amino-2-[hydroxymethyl]-1,3 propandiol
t-RNA	Transfer Ribonucleic Acid
u	Unit
u/ml	Unit per milliliter
V	Volt
w/v	Weight for volume
µg	Microgram
µg/ml	Microgram per milliliter
µl	Microliter
µM	Micromolar

CHAPTER 1. INTRODUCTION

1.1 Importance of Trace Elements to Human Health

Micronutrients play an important role in balancing the human health throughout their lives. Some vitamins namely, Vitamin A, Vitamin B, Vitamin D, Vitamin C, and Vitamin E; and some minerals, namely, calcium, magnesium, iodine, lithium, nickel, copper, zinc, iron, and selenium can be counted as examples.

Moreover, some of the vitamins and trace elements mentioned above have key roles in balancing the antioxidant status of the body. Among the vitamins, Vitamin C and Vitamin E, and among the trace elements, copper, zinc, iron, and selenium, are antioxidants.

Vitamin C is a water soluble vitamin. It is essential for healthy teeth and bones and helps heal wounds and scar tissue, builds resistance to infection, aids in the prevention and treatment of common cold, gives strength to blood vessels, and aids in iron absorption. This vitamin is required for the synthesis of collagen, which holds tissues together. Moreover, it has immunomodulating functions and thus influences the susceptibility of the host to infectious diseases and the course and outcome. Furthermore, its antioxidant protective role has been documented (Bhaskaram, 2002; Gaetke and Chow, 2003). Vitamin C supplementation has been shown to prevent lipid peroxidation in human fibroblast cultures (Anane and Creppy, 2001). In addition, it is involved in downmodulating human granulocyte macrophage-colony-stimulating factor (GM-CSF) signaling by suppressing GM-CSF dependent phosphorylation of the signal transducer and activator of

transcription 5 (Stat-5) and mitogen-activated protein kinase (MAPK; Juan *et al.*, 2002).

Vitamin E, the main antioxidant scavenger, prevents the initiation and progression of oxidative damage by being incorporated into cellular membranes and acting as a potent hydroxyl radical ($OH^{\cdot}$) scavenger. The protective role of Vitamin E in oxidative stress induced DNA damage may be mediated through inhibition of free radical formation by its scavenger role. It is capable of reverting the copper induced oxidative stress; hydrogen peroxide (H_2O_2) induced activation of transcription factor, nuclear factor kappaB (NFκB), and activating protein-1 (AP-1) binding activity. Individuals with impaired immune responses associated with viral infections have been reported to have low serum vitamin E levels. There is an inverse correlation between Vitamin E levels and tumor incidence. Vitamin E causes differentiation of dedifferentiated malignant cells indirectly through adenylate cyclase, which results in the release of transforming growth factor beta (TGF-β) that inhibits growth of malignant cells (Bendich, 2001; Packer *et al.*, 2001, Bulger and Maler, 2003; Drisko *et al.*, 2003; Fariss and Zhang, 2003; Gaetke and Chow, 2003).

Copper is an essential trace element found in a variety of cells and tissues in small amounts with highest levels in liver. It is illustrated to be associated with macromolecules such as proteins, DNA, enzymes and carbohydrates. Moreover, copper has a role in vital processes due to its function in the action of important cellular enzymes. It acts as a cofactor and is necessary for structural and catalytic properties of enzymes that are required for growth, development, and maintenance. Cytochrome c oxidase, tyrosinase, dopamine beta hydroxylase, and Copper/Zinc superoxidase dismutase (Cu/Zn SOD) are among those enzymes that require copper as a cofactor. The performance and the antioxidative and oxidative status are improved by moderate dietary copper. The level of free copper ions is important in balancing the copper-toxicity and the essentiality. Excess copper ion can lead to oxidative stress by combining with superoxide radical ($^{\cdot}O_2^{\cdot}$) and neuronal degeneration similarly, copper-deficiency leads to oxidative stress due to the inactivation of Cu/Zn SOD and contribute to osteoporosis and ischemic heart disease (Gaetke and Chow, 2003; Klevay, 1998).

Zinc has been shown to be involved in growth. It is found in the biological membranes. The idea of zinc finger domains illustrates its role in gene expression and endocrine function. Moreover, zinc interacts with hormones, namely, somatomedin-c, osteocalcin, testosterone, thyroid hormones, and insulin. Bone contains high concentrations of zinc which is demonstrated to be an essential component of calcified matrix. Vitamin D effect on bone metabolism is also enhanced by zinc through stimulation of DNA synthesis in bone cells. Furthermore, zinc acts on the central nervous system and alters appetite control via altering the responsiveness of receptors to neurotransmitters. Zinc dependent enzymes participate in brain growth, and zinc finger proteins are involved in brain structure and neurotransmission. Zinc is involved in DNA and RNA synthesis, which is related to cell replication, differentiation of chondrocytes, osteoblasts, and fibroblasts, synthesis of collagen, osteocalcin, somatomedin-c, and alkaline phosphatase. Moreover, it takes a part in carbohydrate, lipid, and protein metabolism providing good food utilization. It is also found in the structure of Cu/Zn SOD that acts as an antioxidant enzyme. In addition, zinc-deficiency can lead to maternal and neonate morbidity and mortality, and diarrhea (Salguerio *et al.*, 2002).

Iron is essential for aerobic life. It is part of proteins required for important cellular processes. The proteins that require iron have essential roles in oxygen transport and electron transport, ATP production, DNA synthesis, and other molecular processes. Low solubility as the uncomplexed metal and its propensity to catalyze formation of toxic oxidants are the two problems associated with the utilization of iron in biological systems. Low iron status causes anemia which is a major public problem. Iron-deficiency affects more than 2 billion people and it is estimated that %50 of pregnant women in developing countries, and up to 80% in South Asia have iron-deficiency anemia. High iron ion stores leads to lipid peroxidation in the presence of $OH^{\cdot}$, induces depletion of other minerals, alters immune status, and enhances colon carcinogenesis (Quinlan *et al.*, 2002; Tapiero *et al.*, 2001; Gera and Sachdev, 2002).

Selenium has been illustrated as an essential trace element due to its role in selenoproteins, namely, glutathione peroxidases (Gpxs), iodothyronine

deiodinases, thioredoxin reductases, and selenophosphate synthetases. Thioredoxin reductase has antioxidant and redox regulatory roles in conjunction with thioredoxin (Nordberg and Arner, 2001). Selenophosphate synthetases are involved in selenocysteine synthesis (Low *et al.*, 1995). Iodothyronine deiodinases function in deiodination of thyroid hormones (Burk *et al.*, 2003). The antioxidant role of selenium is related to its necessity for Gpxs, which are involved in removal of hydroperoxides (Flohe *et al.*, 2000). Gpx enzymes work in the presence of selenium and protect cellular macromolecules from the damage induced by oxidative stress. Selenium-deficiency can lead to diseases such as nutritional muscle dystrophy, heart diseases, liver necrosis, and certain cancers. Selenium-toxicity, also known as selenosis, results due to excess levels of selenium. Selenium-toxicity has been documented to cause anemia, leucopenia, gastrointestinal disturbances, hair and nail changes, and neurological manifestations (Tinggi, 2003). Importance of selenium to human health will be discussed in detail in section 1.2.

1.2 Selenium and Human Health

The soil where the plants are grown is the source of selenium that is present in the food chain. Bread, cereals, fish, poultry, and meat are rich in selenium. It functions in antioxidant defense mechanism by getting incorporated into the active site of Gpxs. The National Research Council recommends 50-200 μg of daily intake of selenium for adults, depending on the geographical area. (Schwarz and Foltz, 1999). In China, two selenium-deficiency syndromes, namely, Keshan disease, which is an endemic cardiomyopathy, and Keshin-Beck disease, which is a deforming arthritis disease, have been described (see Diplock, 1994; see Rayman, 2000).

Selenium supplementation to selenium-replete individuals stimulates clonal expansion of activated T cells resulting in enhancement of immune functions (Kiremidjian-Schumacher *et al.*, 1994). Moreover, selenium is necessary for testosterone biosynthesis, the formation and normal development of spermatozoa (Behne *et al.*, 1996), and the regulation thyroid hormone metabolism (Arthur *et al.*, 1996). Furthermore, selenium was shown to protect keratinocytes from

apoptosis resulting from ultraviolet radiation (UVR; Rafferty *et al.*, 2003). Selenium has been shown to sustain the growth of selected human hepatocellular carcinoma cell lines, namely, Hep3B, HepG2, and Huh-7 under serum-free conditions, but the detailed mechanism remained undetermined (Nakabayashi *et al.*, 1982; Baker *et al.*, 1993).

Besides, occurrence, virulence, and disease progression of some viral infections are related to selenium-deficiency (Rayman, 2000). For example, CVB3/0, a normally benign strain of amyocarditic Coxackievirus, becomes virulent under selenium-deficiency. Coxsackievirus recovered from the hearts of selenium-deficient mice and inoculated into selenium-adequate mice caused significant heart damage giving evidence that amyocarditic Coxsackie virus had mutated to a virulent phenotype (Beck *et al.*, 1995). In addition, the discovery that the replication of Human Immunodeficiency virus (HIV) is hindered under selenium supplemented conditions supports the importance of selenium in viral infections (Sappey *et al.*, 1994). In addition to these, protective role of selenium supplementation against hepatitis B and C virus (HBV, HCV) infection, progression, and primary liver cancer has been reported (Yu *et al.*, 1997; Yu *et al.*, 1999).

Inverse relationship has been suggested by epidemiological studies between selenium levels and different cancers including prostate cancer (Duffield-Lillico *et al.*, 2003; Yoshizawa *et al.*, 1998; Willett *et al.*, 1983), lung cancer (Knekt *et al.*, 1998; Kneckt *et al.*, 1990), gastrointestinal cancer (Willett *et al.*, 1983), stomach cancer (Kneckt *et al.*, 1990), squamous esophageal and gastric cardia cancers (Mark et al., 2000), and liver cancer (Corrocher *et al.*, 1986; Yu *et al.*, 1999; Buljevac *et al.*, 1996).

1.3 Intrinsic Molecular Mechanisms Involving Selenium

1.3.1 Glutahione Peroxidases

Elementary selenium and inorganic or organic selenocompounds cannot prevent oxidative stress. Thus, the function of selenium becomes evident when incorporated into enzymes. In eukaryotes four groups of selenoenzymes have been described so far. These are deiodinases, thioredoxin reductases, selenophosphate

synthetase, and Gpxs (Flohe *et al.*, 2000). Gpxs can be grouped into two categories as selenium-independent and selenium-dependent.

Selenium-independent epididymis-restricted glutathione peroxidase 5 protein (Gpx5) lacks selenium in the active site of this enzyme. Thus, unlike the other Gpxs characterized to date, it was suspected that Gpx5 can back up inactive selenium-dependent Gpxs in mice subjected to selenium-deficiency (Vernet *et al.*, 1999). Another similar selenium-independent Gpx activity is observed in Glutathione S Transferase (GST) due to selenium-deficiency (Masukawa *et al.*, 1984; Yang *et al.*, 2002).

Selenium-dependent Gpxs contain selenium at their active site and function only in the presence of selenium. Gpxs catalyze the reduction of hydroperoxides (R: side chain; ROOH) and H_2O_2 to water (H_2O) and alcohol (ROH). Meanwhile, Gpxs oxidizes two molecules of reduced glutathione (GSH) to oxidized form (GSSG), and finally glutathione reductase (Glu-Reductase) uses NADPH as an electron donor for converting GSSH back to GSH as shown in Figure 1.7.1.

1.3.2 Selenium-Dependent Glutathione Peroxidases

Mammalian cells are protected against low levels of oxidative stress by selenium-dependent Gpxs that decompose hydroperoxides and H_2O_2 by using GSH as a hydrogen donor and NADPH as an electron donor (Yan and Harding, 1997; Kinnula *et al.*, 1992) as shown in Figure 1.7.1.

These enzymes have 4 protein subunits (tetrameric), each having one atom of selenium. Selenium is incorporated into the polypeptide chain via a co-translational mechanism as the 21st amino-acid, selenocystein (Bock *et al.*, 1991). Specific anti-codon for selenocysteyl- tRNA$^{(ser)sec}$ recognizes UGA codon in the corresponding mRNA. Contrasting to usual protein synthesis, tRNA is first charged with serine forming seryl-tRNA$^{(ser)sec}$, which is then transformed into selenocysteyl-tRNA$^{(ser)sec}$ by means of selenophosphate (Li *et al.*, 1990).

Selenium availability is necessary for Gpx enzymatic activity and protein synthesis. In HL-60 cells grown in the absence of selenium and in erythrocytes from a selenium-deficient patient, selenium treatment restored Gpx activity and protein levels pointing out a direct relationship between selenium availability, the Gpx enzymatic activity and the Gpx protein levels (Takahashi *et al.*, 1986).

The effect of dietary selenium on Gpx is at the level of regulation of the stability of cytosolic mRNA in rat liver (Christensen and Burgener, 1992). Moreover, tissue specific differential regulation of Gpx by selenium depletion and repletion has been described (Bermano *et al.*, 1995). Differential control of different isoforms of Gpx in hepatoma cell line, H4, at the mRNA stability level has been illustrated (Bermano *et al.*, 1996). Human hepatoma-derived Hep3B cells and hepatoblastoma HepG2 cells exhibited time-dependent decrease and total loss in Gpx activity upon culture in selenium-deficient medium for 10 days (Baker *et al.*, 1993). A co- and/or post-translational control mechanism in addition to the effect on mRNA stability has been suggested under selenium-depleted and -repleted conditions (Toyoda *et al.*, 1989; Baker *et al.*, 1993).

In mammals, at least four isoenzymes of selenium-dependent Gpxs, namely, cytosolic or mitochondrial, phospholipid hydroperoxide, and extracellular Gpx, all with ubiquitous expression pattern have been identified. Table 1.3.2.1 summarizes the selenium-dependent Gpxs, their cellular locations, and their functions. Gpx1/cGpx is cytosolic or mitochondrial and is involved in the reduction of fatty acid hydroperoxides and H_2O_2. If these molecules are not eliminated by Gpxs, a chain reaction of lipid peroxidation can start and damage cellular membranes (Chambers *et al.*, 1986; Esworthy *et al.*, 1997). Overexpression of Gpx in cell lines confers resistancy to oxidative stress both *in vitro* and *in vivo* (Geiger *et al.*, 1991; Mirault *et al.*, 1991; Doroshow, 1995; Cheng *et al.*, 1998). Mice with a null Gpx gene were demonstrated to be sensitive to oxidative stress exposure when compared to the wild type controls (de Haan *et al.*, 1998). Fatty acid hydroperoxides, phospholipid hydroperoxides, and cholestrol hydroperoxides, that are produced in peroxidized membranes and oxidized lipoproteins, can be decomposed by phospholipid hydroperoxide Gpx (Gpx4/PHGpx; Maiorino *et al.*, 1991; Schuckelt *et al.*, 1991). PHGpx is localized in both membrane and cytosolic fractions (Imai *et al.*, 1998; Flohe *et al.*, 2000). Gpx1 and PHGpx are found in

nearly all tissues of mammalians, the former being mainly in erythroctes, kidney, and liver and the latter being mainly in renal epithelial cells and testis. On the other hand, cytosolic Gpx (Gpx2 or Gpx-G1; Chu *et al.,* 1993; Chu and Esworthy, 1995) and extracellular Gpx (Gpx3 or Gpx-P; Takahashi *et al.*, 1987; Yoshimura *et al.*, 1991; Chu *et al.*, 1992) are weakly detected in various tissues excluding gastrointestinal tract and kidney, respectively.

Table 1.3.2.1: Selenium-Dependent Glutathione Peroxidases.

Name	**Abbreviation**	**Body Localization**	**Cellular Localization**	**Function**
Cytosolic or classical Gpx	cGpx or Gpx1	Ertythrocytes, kidney, liver	Cytosolic, mitochondrial	Reduces fatty acid hydroperoxides, H_2O_2
Cytosolic Gpx	Gpx2 or Gpx-G1	Gastrointestinal tract	Cytosolic	Reduces H_2O_2
Extracellular Gpx	Gpx3 or Gpx-P	Kidney	Extracellular	Reduces H_2O_2
Phospholipid hydroperoxide Gpx	PHGpx or Gpx4	Renal Epithelial cells, testes	Cytosolic, membrane	Reduces fatty acid, phospholipid, cholesterol hydroperoxides

1.4 Intracellular and Extracellular Sources of Oxidative Stress

Several environmental stresses can influence the natural balance between life and death during the lifetime of an organism. Reactive oxygen species (ROS), that are the reactive byproducts of oxygen, are amongst the most threatening and most potent ones that organisms face. ROS are continually generated under normal physiological conditions due to aerobic metabolism. Also, pathobiochemical toxic insults can lead to ROS generation. ROS consists of free radicals such as $^{\cdot}O_2^{\cdot}$, $OH^{\cdot}$, and peroxyl radical ($ROO^{\cdot}$) and nonradical H_2O_2 and singlet oxygen ($^1O_2$). These species are transient with half-lives ranging from nanoseconds to hours depending on the species. They can react with cellular macromolecules such as proteins, lipids, nucleic acids, and carbohydrates due to their high chemical reactivity (Stahl and Sies, 2002). ROS can be eliminated by internal defense mechanisms such as Gpx, superoxide dismutase (SOD), catalase (CAT), and GST, and also by dietary defense mechanisms such as Vitamin E, selenium, and copper, zinc supplementation. Gpx activity has been explained in section 1.3 in detail. SOD, CAT and GST will be explained in section 1.7.

Both enzymatic and non-enzymatic sources can be involved in ROS generation. Mitochondria, endoplasmic reticulum (ER), membranes with ROS generating enzymes, and peroxisomes are considered to be cellular sources of ROS (See Freeman and Crapo, 1982).

Autooxidation of the reduced components of the electron transport chain that is localized to inner mitochondrial membrane is an active and continuous source of ROS during cellular respiration. $^{\cdot}O_2^{\cdot}$ and H_2O_2 are the free radical intermediates formed during this process (Fernandez-Checa *et al.*, 1998). If these are not eliminated by antioxidant mechanisms, in the presence of ferrous iron (Fe^{2+}) or cuprous ion (Cu^{+}), H_2O_2 is rapidly reduced to $OH^{\cdot}$, the most reactive oxygen species. Fe^{2+} and Cu^{+} are oxidized to ferric iron (Fe^{+3}) and cupric ion (Cu^{2+}), respectively, as shown Figure 1.4.1. $OH^{\cdot}$ can directly attack nucleic acids leading to DNA modifications (Zigler *et al.*, 1985).

Moreover, as in the case of Fenton reaction, if $^{\cdot}O_2^{\cdot}$ and H_2O_2 are not eliminated by cellular antioxidant defense mechanisms, metal ions such as iron and copper are

reduced and they catalyze the formation of $OH^{\cdot}$. The so called metal catalyzed Haber-Weiss reaction is shown in Figure 1.4.2 (Rigo *et al.*, 1977).

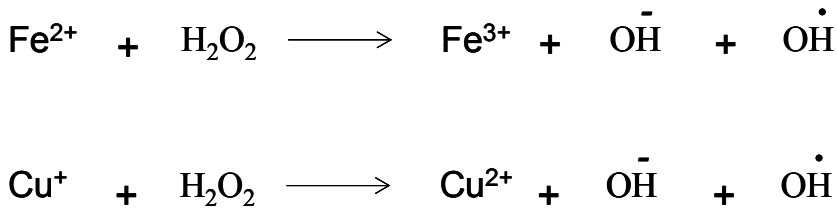

Figure 1.4.1: Fenton Reaction. Metal catalysts iron (upper) or copper (lower) catalyzes the conversion of H_2O_2 to $OH^{\cdot}$ if H_2O_2 is not eliminated by defense mechanisms.

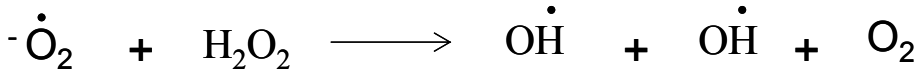

Figure 1.4.2: Haber-Weiss Reaction. Metal ions catalyze the formation of $OH^{\cdot}$ if $^{-}O_2^{\cdot}$ and H_2O_2 are not eliminated.

Smooth ER contains detoxifying enzymes involved in detoxification of lipid-soluble drugs and other toxic metabolic products. Oxidization of unsaturated fatty acids and xenobiotics and finally reduction of molecular oxygen (O_2) to $^{-}O_2^{\cdot}$ and/or H_2O_2 are catalyzed by cytochrome P-450 and b5 (Aust *et al.*, 1972; Capdevila *et al.*, 1981; See Freeman and Crapo, 1982). Growth factor and/or cytokine stimulated oxidant generation may be due to the oxidases localized to the plasma membranes. NADPH oxidase system has been identified in fibroblasts and leukocytes (Meier *et al.*, 1991). NADPH-dependent H_2O_2 generation in plasma membranes is the most characterized oxidation and is involved in host defense against invading microorganisms (Meier *et al.*, 1989; Krieger-Brauer and Kather, 1995).

H_2O_2 generating enzymes in peroxisomes include glycolate oxidase, D-amino acid oxidase, urate oxidase, L-α-hydroxyacid oxidase, and fatty acyl-CoA oxidase. Peroxisomal reduction reactions are important mainly for liver and kidney cells since toxic molecules such as ethanol are detoxified in the peroxisomes of these organelles. β-oxidation of fatty acids is also carried out in peroxisomes and mitochondria.

As well as membrane-associated oxidases, soluble enzymes such as xanthine oxidase, aldehyde oxidase, dihydroorotate dehydrogenase, flavoprotin dioxygenase are sourses of ROS generation. Moreover, ROS can be produced by autooxidation of small molecules such as dopamine, flavins, epinephrine, and hydroquinones (See Thannickal and Fanburg, 2000).

In addition to all above, ligand-induced ROS production by a variety of cytokines and growth factors in nonphagocytic cells has been illustrated (Thannickal and Fanburg, 2000). Intracellular concentration of H_2O_2 has been shown to increase transiently following stimulation of rat vascular smooth muscle cells and primary lung fibroblasts (Sundaresan *et al.*, 1995; Thannickal and Fanburg, 1995). Also, myofibroblast differentiation upon ROS generation due to TGF-β stimulation in primary lung fibroblasts has been demonstrated (Thannickal and Fanburg, 1995; Thannickal *et al.*, 2000).

Moreover, aflatoxinB1 (AFB1) contaminated foods (Jayashree and Subramanyam, 2000), alcohol consumption, viral infections (Perez and Cederbaum, 2003; Rigamonti *et al.*, 2003), and exposure to metal ions such as copper, iron, zinc (discussed in section 1.1), UVR (Butts *et al.*, 2003) can be counted among the extracellular sources of ROS. There is indirect evidence that oxidative stress may play a role in genesis of hepatitis B virus (HBV) induced hepatocellular carcinoma (HCC; Hagen *et al.*, 1994). Exposure to such external factors leads to alterations in DNA.

1.5 Cellular ROS Toxicity

Oxidative stress is associated with several disease conditions such as aging, inflammation, carcinogenesis, ischemia-reperfusion, AIDS, Parkinson's

Huntington's, Alzheimer, familial amyotrophic lateral sclerosis, and cataract formation in the eye (Chandra *et al.*, 2000).

Cellular macromolecules such as proteins, DNA, and lipids are targets of free radicals, which are not eliminated by cellular antioxidant defense mechanisms. Free radicals alter cellular functions of the macromolecules permanently, leading to severe consequences that are harmful to the organism (Wells and Winn, 1996).

DNA modifications such as altered purine and pyrimidine bases represent the main class of $OH^{\cdot}$ mediated DNA damage that includes oligonucleotide strand breaks, DNA-protein cross-links and abasic sites generated in mammalian chromatin *in vitro* and *in vivo* (Dizdaroglu, 1992; Cadet *et al.*, 1999). 8-hydroxy-deoxyguanosine (8-OH-dG) is formed upon oxidation of guanine by $OH^{\cdot}$ consequent to X-ray and gamma irradiation (Kasai *et al.,* 1986). These alterations in DNA lead to mutagenesis (See Ames, 1983) and carcinogenesis (See Floyd, 1990). Formation of 8-OH-dG is involved in aging (Fraga *et al.*, 1990), diabetes mellitus (Dandona *et al.*, 1996), inflammatory diseases (See Ames, 1983), and progression of a severe chronic hepatitis in liver cancer (Sipowicz *et al.*, 1997). This alteration can be repaired specifically with endonuclease DNA glycosylase (Chung *et al.*, 1991).

Polyunsaturated fatty acids are targets for lipid peroxidation. The peroxidation is induced when $OH^{\cdot}$ captures a hydrogen atom from methylene carbon in the polyalkyl chain of fatty acid ($R\text{-}CH_2\text{-}$) resulting in an alkali radical ($R\text{-}C^{\cdot}H\text{-}$). This radical can react with O_2 to create peroxyl radical ($ROO^{\cdot}$). Membrane proteins can be altered upon further reaction of peroxyl radicals with other peroxyl radicals. Furthermore, a chain reaction occurs if they capture hydrogen molecule from adjacent fatty acids leading to the formation of more lipid peroxides as shown in Figure 1.5.1 and Figure 1.7.1 (Cheeseman, 1993; Gate *et al*., 1999). Malondialdehyde (MDA) is a highly reactive product of lipid peroxidation (Draper and Hadley, 1990). It can attack free amino-group of phospholipids, proteins, and lipids and consequently, form inter- and intra-molecular 1-amino-3-iminopropene bridges. By this way, cellular macromolecules are structurally altered (Halliwell and Gutteridge, 1999), leading to autoimmune response (Kergonou *et al.*, 1987). Increased levels of lipid peroxidation have been reported

in diabetes (Sato *et al.*, 1979), apoplexy (Mori *et al.*, 1990), hyperlipemia (Esterbauer *et al.*, 1990), atherosclerosis (Plachta *et al.*, 1992), and liver diseases (Rouach *et al.*, 1997). Besides, during inflammation, enzymes such as lipooxygenases and cyclooxygenases produce specific fatty acyl peroxides (Miller *et al.*, 1985) and cholesterol and fatty acid moieties of the plasmatic low-density lipoproteins (LDL). This lipid structures can also be oxidized in the presence of oxidative stress. The oxidation of LDL is observed to be involved in atherosclerosis (Steinberg *et al.*, 1989; Galle *et al.*, 1995).

Like nucleic acids and lipids, proteins are also attacked by free radicals. Oxidative modification of proteins can be toxic to the cells if the ROS generated overrides the physiological levels of ROS required for intracellular ROS mediated signaling. $^1O_2$ and $OH^{\cdot}$ attacks are common *in vivo* mechanisms that contribute to damage on proteins by protein crosslinkings and cleavage of the peptide bonds to macromolecules (Cohen *et al.*, 1998; Prinsze *et al.*, 1990). There are studies demonstrating that via metal-catalyzed oxidative reactions that can occur in metal binding sites of the proteins, histidine can be oxidized to 2-oxohistidine (Lewisch and Levine, 1995). Also hypochlorous acid causes oxidation of thyrosine to 3-chlorotyrosine (Domigan *et al.*, 1995). Modification of proteins by oxidation of cystein residues, formation of intra-molecular disulfide linkages, dithyrosine formation, and metal catalyzed oxidation of the proteins are the mechanisms that result in oxidative modification of proteins (Thannickal and Fanburg, 2000). Disorders associated with protein oxidation reported until now are atherosclerosis, ischemia-reperfusion injury, and aging (See Stadtman, 1992; Berliner and Heinecke, 1996).

A general overview of ROS toxicity on cellular macromolecules is given in Figure 1.5.1. The figure summarizes the effects of ROS on DNA, lipids, and proteins (Mates and Sanchez-Jimenez, 1999).

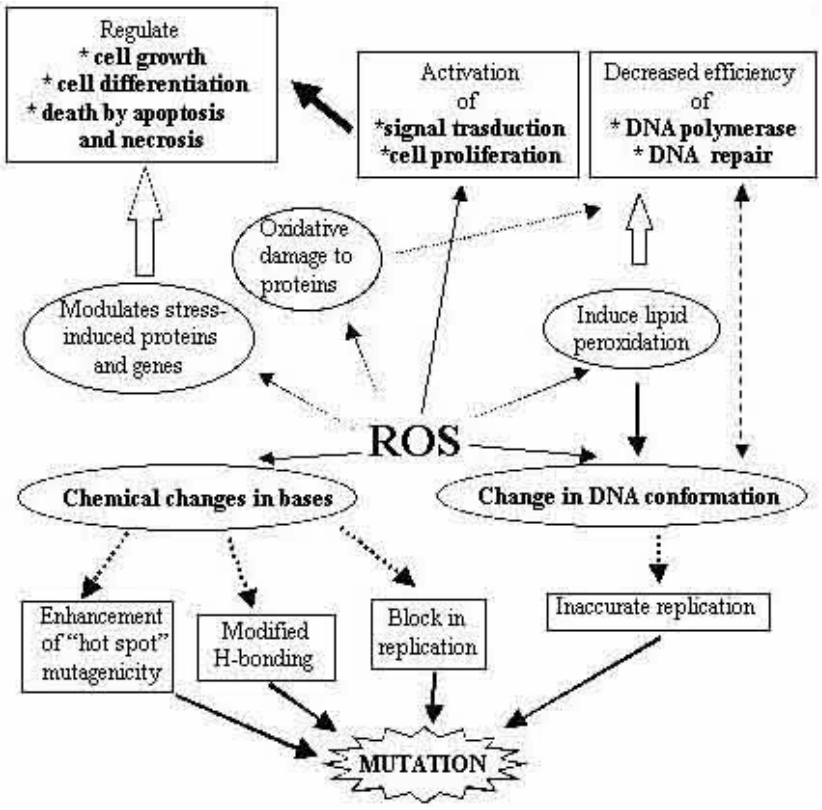

Figure 1.5.1: ROS Toxicity (Mates and Sanchez-Jimenez, 1999).

1.6 Signaling Molecules Targeted by ROS

Despite the mutagenic and carcinogenic effects of ROS in case of excess, there is a huge number of signal transduction pathways regulated by ROS, but the specific molecules targeted by ROS are not yet very apparent (Thannickal and Fanburg, 2000).

Receptor kinases and phosphatases may be targets for oxidative stress. Ligand independent activation of growth factor receptors in response to UV has been documented and related to ROS. Another study recently demonstrated that exposure of human keratinocytes to $^1O_2$ resulted in rapid loss of EGF receptor, which indicates that oxidative stress produced by $^1O_2$ rapidly disrupts EGF receptor mediated signaling (Zhuang *et al.*, 2003). In addition, H_2O_2 induced

phosphoinositide 3-kinase (PI3K) and Akt (also known as protein kinase B) activation has been reported to be achieved through PI3K membrane recruitment to its substrate site, thereby enabling PI3K to maximize its catalytic efficiency (Qin and Chock, 2003). Another recent work illustrates that Akt activation by H_2O_2 is dependent on the activation of EGF receptor signaling (Wang *et al.*, 2000).

Due to the fact that activation of mitogen activated protein kinase (MAPK) pathways depends on mitogen and stress activated signals, redox regulation of these pathways is not surprising. There are studies showing exogeneous ROS-mediated ERK activation to be an upstream event at the level of growth factor receptors and Src kinases, there are some others suggesting oxidant induced inactivation of protein tyrosine phosphatases (PTPs) or protein phosphatase A. There are limited studies showing the endogenous ROS dependent activation of ERK MAPK pathway (Thannickal and Fanburg, 2000). Many studies demonstrate c-Jun N-terminal kinases (JNK) activation to be linked to cell death or apoptosis, while others suggest pro-survival function for JNK upon oxidative stress (Martindale and Holbrook, 2002). Pro-survival and apoptotic functions of protein kinase C (PKC) have been documented (Martindale and Holbrook, 2002).

P53 activation through environmental stress depends largely on the posttranslational mechanisms that enhance its stability and increase DNA binding activity. Oxidative stress dependent direct DNA damage activates p53. NFκB dependent upregulation of p53 upon H_2O_2 exposure has been documented. Furthermore, p53 protein has been illustrated to be phosphorylated by both JNK and p38 and stabilized under oxidative stress conditions (Fuchs *et al.*, 1998; Bulavin *et al.*, 1999). P53 also represses the expression of anti-apoptotic factor, Bcl-2, and enhances the expression of proapoptotic factor, Bax and antioxidant enzyme Gpx upon oxidative stress (Martindale and Holdbook, 2002).

NFκB (Nuclear factor kappa B) is an oxidant responsive transcription factor that is involved in the expression of genes that have roles in immune and inflammatory responses. It is suggested that redox-regulated effect occurs downstream from inhibitor of kappaB (IκB) kinases at the level of degradation of IκB. Also AP-1 has also been considered as an oxidant responsive transcriptional complex. Both

exogenous ROS and ligand induced ROS have been implicated in AP-1 activation (Thannickal and Fanburg, 2000).

Non-receptor tyrosine kinases such as Src kinases and Janus kinases (JAK) have been reported to be activated by oxidative stress. As in the case of receptor tyrosine kinases, mostly the studies are performed by exogenously added oxidants (Thannickal and Fanburg, 2000).

The molecules targeted by ROS are summarized in Figure 1.6.1.

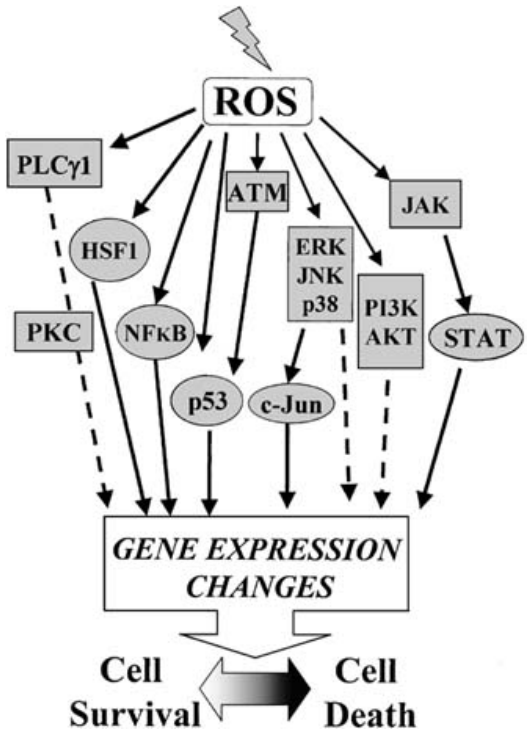

Figure 1.6.1: Signaling molecules targeted by ROS (Martindale and Holdbook, 2002).

1.7 Antioxidants against Oxidative Stress

ROS are generated in a stepwise manner by the reduction of one electron of oxygen each time. Oxidative stress results following disturbance of the balance between intracellular pro-oxidant to anti-oxidant status of the cell consequent to the inefficiency of cellular anti-oxidant defense mechanisms to cope with ROS (Dizdaroğlu, 1992).

Antioxidants are directly or indirectly involved in maintaining intracellular balance between pro-oxidant and anti-oxidant levels. These antioxidants that favor the anti-oxidant levels in the cell are biologically important molecules. They include vitamin C (ascorbic acid), vitamin E (α-tocopherol), vitamin A, β-carotene, metallothionein, polyamines, melatonin, NADPH, adenosine, coenzyme Q-10, urate, ubiquinol, polyphenols, flavonoids, phytoestrogens, cystein, homocysteine, taurine, methionine, S-adenosyl-L-methionine, resveratrol, nitroxides, thioreductase, nitric oxide synthase, heme oxygenase-1, Gpxs, superoxide dismutases (SODs), catalase (CAT) (Matés, 2000), and GST (Arthur, 2000). The detailed explanation for Gpxs was given in 1.3.2. SOD, CAT and GST will be discussed in section 1.7.1 and the dietary defense mechanisms including Vitamin E, selenium, and Copper/Zinc in section 1.7.2.

1.7.1 Internal Defense Mechanisms

1.7.1.1 Superoxide Dismutases

The $^{\cdot}O_2^-$ radical generated during cellular respiration is dismutated rapidly with SOD enzymes generating H_2O_2 as shown in Figure 1.7.1. Four classes of SOD, namely, manganese SOD (Mn-SOD), Cu/Zn SOD, extracellular SOD (EC-SOD), and nickel SOD (Ni-SOD) have been identified so far (McCord and Fridovich, 1969; Weisiger and Fridovich, 1973; Yost and Fridovich, 1976; Youn *et al.*, 1996). The function of SOD is to convert $^{\cdot}O_2^-$ to H_2O_2 by catalyzing one-electron redox cycle of superoxide. Mn-SOD is localized in mitochondrial matrix while Cu/Zn-SOD is located into intracellular cytoplasmic compartments and also associated with mitochondrial and peroxisomal membranes (Kira *et al.*, 2002; Zelko *et al.*, 2002). Mn-SOD is a nuclear-encoded main antioxidant enzyme that

removes $^{-}O_2^{\cdot}$ produced in mitochondria during respiration (Guan *et al.*, 1998) and has been reported to be a tumor suppressor gene (Mates, 2000). Studies with SOD knockout mice revealed that Mn-SOD was essential for life while copper/zinc SOD was not (Reaume *et al.*, 1996). EC-SOD is a secretory Cu/Zn containing SOD that is the primary antioxidant in blood vessel interstitium and the only extracellular enzyme that reduces $^{-}O_2^{\cdot}$ (See Sentman *et al.*, 1999; Enghild *et al.*, 1999).

1.7.1.2 Catalase

Catalase is a ubiquitous anti-oxidant enzyme that is predominantly localized to peroxisomes of the cells and decomposes H_2O_2 to H_2O and O_2 as shown in Figure 1.7.1. CAT confers protection against severe oxidative stress with a high affinity to H_2O_2. Due to its efficiency towards H_2O_2, it cannot be saturated by at any concentration of H_2O_2 (Kinnula *et al.*, 1992; Yan and Harding, 1997; Mates, 2000). The cells are protected against H_2O_2 generated within them by this enzyme.

1.7.1.3 Gulutathione S Transferases

GSTs play an important role in the detoxification and elimination of xenobiotics. It has been demonstrated that these enzymes have an additional ability to reduce lipid hydroperoxides (ROOH) by selenium-independent means via their selenium-independent Gpx activity using GSH as a hydrogen donor as shown in Figure 1.7.1. It has been shown that GST contributes to selenium-independent Gpx activity in the liver of selenium-deficient rats (Lawrence *et al.*, 1978). In addition, dietary selenium-deficiency produced increased activity of GST suggesting that GST activity is regulated by dietary selenium (Masukawa *et al.*, 1984). Moreover, increased resistance to oxidative stress has been documented in cells transfected with (Glutathione S transferase alpha) GSTα, which is among one of the five GST gene families (Zimniak *et al.*, 1997). Previously it has been reported that patients with primary biliary cirrhosis, primary sclerosing cholongitis, and chronic hepatitis B had significantly elevated levels of GST-alpha (Mulder *et al.*, 1997).

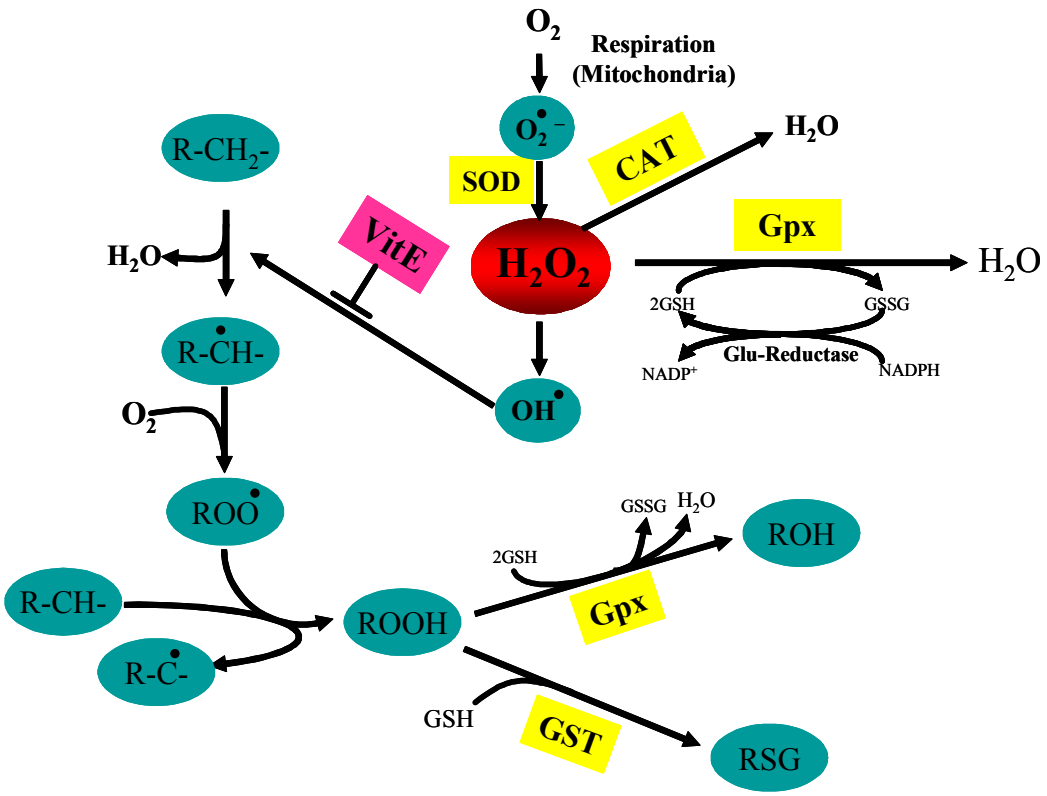

Figure 1.7.1: Internal Defense Mechanisms against ROS. SOD is involved in the elimination of $^{\bullet}O_2^{-}$. Gpx and CAT are involved in the elimination of H_2O_2, and Gpx and GST are involved in the elimination of lipid peroxides (ROOH) avoiding a chain reaction (Halliwell and Gutteridge, 1999). Vitamin E prevents lipid peroxidation by scavenging $OH^{\bullet}$ (Claycombe and Meydani, 2001).

1.7.2 Dietary Defense Mechanisms

1.7.2.1 Vitamin E Supplementation

The importance of Vitamin E for human health has been described in section 1.1. Vitamin E supplementation has been suggested a valuable therapy in case of its deficiency. Vitamin E supplementation could prevent free radical induced damages to the tissues in the patients due to the radical scavenger role. Vitamin E

supplementation to healthy elderly people has been illustrated to enhance their immune responses (Meydani *et al.*, 1997; Meydani *et al.*, 1998). Vitamin E supplementation has been documented to delay early progression of arteriosclerosis in heart transplant patients (Liu and Meydani, 2002). Pathogenesis of rheumatoid arthritis and systemic lupus erythematosus has been found to be associated with low serum Vitamin E concentrations (Comstock *et al.*, 1997). Vitamin E supplemented type I diabetic children have been documented to restore GSH and MDA to normal levels (Jain *et al.*, 2000). Vitamin E protects humans against atherosclerosis, ischemic heart disease, and development of some type of cancers such as breast, prostate, and leukemia (Ricciarelli *et al.*, 2001). The lowered vitamin E levels in patients with viral hepatitis have been documented (von Herbay *et al.*, 1996; von Herbay *et al.*, 1997). High-dose supplementation of Vitamin E has been used as a safety therapy. Vitamin E is absorbed in gastrointestinal tract similar to other lipids. The primary reported side effects of high dose of Vitamin E supplementation includes breast soreness, thrombophlebitis, gastrointestinal disturbances, and depression of Vitamin K dependent coagulation factors when used with other anticoagulation agents (Bulger and Maier, 2003).

1.7.2.2 Selenium Supplementation

The importance of selenium for human health and supplementation has been described in section 1.2. In addition to those, more clinical examples related to selenium-deficiency will be provided in this section. Lowered selenium status has been observed in asthma patients and it is suggested that selenium supplementation might be beneficial to such patients, which may be at risk of selenium-deficiency (Kadrabova *et al.*, 1996). Moreover, in patients with viral infections, selenium-deficiency is associated with skeletal muscle disorders manifested by muscle pain, fatigue, and proximal weakness (Chariot and Bignani, 2003). Severe selenium-deficiency has been reported to result in a high incidence of thyroiditis due to a decreased activity of selenium-dependent Gpx activity within thyroid cells. In those cases, selenium substitution may improve the inflammatory activity in patients with autoimmune thyroiditis, especially in those with high activity (Gartner *et al.*, 2002). Decreased plasma selenium and Gpx

activity has been reported in patients with systemic inflammatory response syndrome and sepsis. Selenium supplementation seems to improve the outcome of those patients, but the results should be improved (Berger *et al.*, 2001). Intensive oxidative stress is observed in the blood of patients with multiple sclerosis. Supplementation of patients with selenium improves the health of patients (Syburra and Passi, 1999). Moreover, selenium supplementation has been reported to have immunoenhancing effects in humans by stimulating cytotoxic lymphocytes and natural killer cells (Kiremidjian-Schumacher *et al.*, 1994). In addition to these, protective role of selenium supplementation against hepatitis B and C virus (HBV, HCV) infection, progression, and primary liver cancer has been reported (Yu *et al.*, 1997; Yu *et al.*, 1999). In contrast to its advantages following supplementation, high doses of selenium has been reported to cause gastrointestinal tract disorders, weakness, neurological manifestations, hypochromic anemia, and leucopenia (Tinggi, 2003).

1.7.2.3 Copper and Zinc Supplementation

Safe and adequate daily intake of dietary copper has been reported to be 1.5 to 3.1 mg for adults. Individuals with copper-deficiency show hypercholesterolemia, abnormal electrocardiograms, and hypertension. The copper is required by obese women during weight loss (Klevay, 1998). Dietary copper-deficiency contributes to high blood pressure, enhancement of inflammation, anemia, reduced blood clotting and arteriosclerosis and consequently may impair cardiovascular health (Saari and Schuschke, 1999). Documentations reveal that copper-deficiency occurs secondary to gastric resection, unsupplemented total parenteral nutrition, high levels of zinc intake, or general malnutrition. Although copper has significant roles in the organism and in the immune system, the molecular mechanism for copper-deficient neutropenia is not known (Percival, 1995). Other groups demonstrated that early clinical sign of copper-deficiency is a reduction in the number of circulating neutrophils. Moreover, the growth of copper-deficient infants recovering from malnutrition is improved by copper-supplementation (Castillo-Duran and Uauy, 1988). Copper-deficiency can cause an X-linked Menkes syndrome, which is related to the deficiency of copper-dependent enzymes (Camakaris *et al.*, 1999). In contrast to these, elevated concentrations of

copper are harmful for health. Copper-toxicity results in liver cirrhosis, damage to renal tubes, and the brain. Chronic copper-toxicity was observed in patients receiving dialysis via copper tubing. Copper-contaminated foods and water lead to gastrointestinal symptoms (Gaetke and Chow, 2003). Moreover, free copper ions take part in the formation of ROS as explained in section 1.4.

Zinc-deficiency leads to anorexia (loss of epitetite), hypogeusia (loss of taste), poor growth, alopecia, and delayed sexual maturation. Zinc supplementation has been reported to improve gastrointestinal tract diseases. A recent study demonstrated that zinc-supplementation in children with acute diarrhea had a reduction of continuing diarrhea. Zinc is essential for growth and development, DNA synthesis, neurosensory functions, and cell-mediated immunity (Duggan *et al.*, 2002). Another recent work showed that zinc treatment of children with growth dysfunction appeared to ameliorate the Cu/Zn-SOD activity which was increased significantly during growth retardation (Kocaturk *et al.*, 2002). Zinc-deficiency is a common nutritional disorder in the elderly. In those people, copper, iron, and lipoprotein status should be monitored if long-term zinc supplementation is required, since they may be affected by the zinc supplementation (Stiles and Boosalis, 1995). Zinc intake is reduced in elderly people, however, its deficiency and effects on cell-mediated immunity of the elderly have not been well documented. Zinc supplementation has been shown to overcome zinc-deficiency and normalize plasma copper levels. Moreover, serum thymulin activity, Interleukine-1 (IL-1) production significantly increases after supplementation. Improvement in response to skin-test antigens and taste acuity was observed after zinc supplementation (Prasad *et al.*, 1993).

1.8 Oxidative Stress and Cancer

A crucial step in carcinogenesis is DNA mutation which is one of the outcomes of oxidative base lesions. Elevated levels of oxidative DNA lesions have been noted in many tumors, suggesting oxidative stress induced damage in the etiology of cancer. Oxidative mechanisms have been demonstrated to possess a potential role in the initiation, promotion, and malignant conversion (progression) stages of carcinogenesis. Given that cancer risk increases with age and is associated with an

accumulation of DNA damage, oxidative DNA damage has been investigated in cancer.

Lesions such as 8-OH-dG are established biomarkers of oxidative stress. The potential mutagenic capacity of such lesions in mammalian cells suggested them as intermediate markers of cancer. G:C → T:A transversions that are potentially derived from 8-OH-dG have been observed *in vivo* in the ras oncogene and the p53 tumor suppressor gene in lung and liver cancer. Also C:C → T:T substitutions in the absence of UV in tumors have been identified as signature mutations for ROS. These findings support the proposal of implication of oxidative damage in carcinogenesis (Cooke *et al.*, 2003).

Due to elevated ROS levels, transcription factors and their corresponding genes are permanently activated, which, coupled with increased DNA damage, creates a selection survival advantage for a malignant phenotype seen in cancer (Toyokuni, *et al.*, 1995). It is important to note that the nuclei of undifferentiated proliferating stem cells must be affected by oxidative stress for the initiation and progression of cancer. Current analytical procedures will not reflect lesion levels in the most important target cells due to the fact that tissue samples from tumors and normal cells will represent a heterogeneous mixture of differentiated and undifferentiated cells with the former likely to predominate (Cooke *et al.*, 2003).

The implication of ROS in tumor formation has been mostly studied indirectly. For example, chemical promoters can generate oxidative stress and antioxidants can inhibit promotion; therefore, ROS are involved in promotion. It is possible that the antioxidants themselves may allow clonal expansion and tumor promotion by protecting initiated cells from excessive oxidant toxicity and apoptosis that would otherwise kill them (Cooke *et al.*, 2003). Finally, in linking oxidative stress with cancer promotion, it should be kept in mind that biomolecules other than DNA may be oxidatively modified and that these may have a significant effect on the carcinogenesis as mentioned in section 1.5 and 1.6.

1.9 Selenium and Cancer

There is growing evidence supporting the association between low dietary intakes of selenium with an increased risk of certain cancers. Moreover, protective effect of selenium supplementation has been observed on the overall incidence of prostate cancer (Yoshizawa *et al.*, 1998; Duffield-Lillico *et al.*, 2003). The strong association between low serum selenium levels and cancer was evident for gastrointestinal and prostatic cancers (Willett *et al.*, 1983). The association between the serum selenium level and the subsequent incidence of cancer was investigated in a group of Finnish people. Low serum selenium levels were associated with an increased risk for cancers, especially stomach and lung, among men (Knekt *et al.*, 1990). Also another study demonstrated that very low selenium status may contribute to the risk of lung cancer (Knekt *et al.*, 1998). Populations with low selenium intake have been documented to have low serum selenium levels and are at a high risk for squamous esophageal and gastric cancers (Mark *et al.*, 2000). Serum selenium concentration and the Gpx activity were positively correlated with the incidence of liver cancer (Corrocher *et al.*, 1986). The inverse association between plasma selenium levels and HCC has been shown to appear in a group of patients especially among cigarette smokers (Yu *et al.*, 1999). Another research also points out the protective role of selenium in the patients with liver cirrhosis and hepatocellular carcinoma, and the potential need of selenium supplementation in these patients (Buljevac *et al.*, 1996).

1.10 Signaling Molecules Targeted by Selenium Supplementation

In addition to the importance of selenium in certain diseases, the essentiality of selenium in the clonal growth of human fibroblasts (McKeehan *et al.*, 1976), human hepatoma cells lines with differentiated functions (Nakabayashi *et al.*, 1982), and mammary tumor cell lines (Barnes *et al.*, 1979) has been clearly demonstrated.

Until recently, the signaling targets that are regulated by selenium availability were largely unknown. There is accumulating data related to the target proteins of selenium, which take part in important signaling pathways.

It has been recently documented that selenite suppresses both the JNK and stress-activated protein kinase (SAPK) as well as the p38 mitogen-activated protein kinase pathway in human embryonic T cells, 293T (Park *et al.*, 2000a). Furthermore, selenium also inhibited caspase 3 activity in embryonic kidney cells (Park *et al.*, 2000b) and activated MAPK in human hepatocytes and adipocytes acting as an insulin-mimetic agent (Stapleton *et al.*, 1997). These proteins are regulated through the redox regulation of their active site cysteine residues. A very recent paper described the activation of PI3K/Akt kinase pathway and inactivation of caspase 3 and apoptosis signal regulating kinase 1 (ASK1) in selenium supplemented Huh-7 cells, which blocked apoptosis and promoted cell survival. The activation of focal adhesion kinase (FAK), and Rac1, upstream inducers of PI3K, have been suggested to have a role in the activation of PI3K pathway in this study (Lee *et al.*, 2003).

1.11 Selenium-Deficiency and Liver Diseases

Selenium has been reported to be protective against dietary necrotic liver degeneration in rats long ago (Schwarz and Foltz, 1999). Since then, the reports pointing out the importance of selenium in prevention of hepatic disease conditions with different etiologies have been accumulating.

1.11.1 Selenium-Deficiency, Keshan Disease and Liver Disorders

Animal and human studies have revealed the existence of an association between certain liver diseases and selenium-deficiency. In addition, it is well documented that in Asia and Africa, dietary selenium-deficiency is associated with a cardiomyopathy known as Keshan disease. Adults dying of Keshan disease had diagnostic lesions not only in the cardiovascular system and skeletal muscle but also in the liver. Varying degrees of focal biliary cirrhosis were identified in 50%

of the Keshan disease autopsies, and 5% developed severe lobular cirrhosis (Wallach *et al.*, 1990).

In animal studies, pigs that suffer from Hepatosis Dietetica have been documented to have low hepatic selenium concentrations (Moir and Masters, 1979). In addition, pigs with mulberry heart disease showed low hepatic selenium concentration. It is suggested that the low selenium status together with vitamin E-deficiency increases oxidative stress and thus contributes to the development of oxidative damage in pigs (Korpela, 1990). Besides, in selenium and vitamin E-deficient pigs with microangiopathy, hepatic iron concentration has been significantly increased. Increase in iron concentration might have promoted oxidative stress and thus contributed to the development of oxidative damage (Korpela, 1990).

The importance of selenium in antioxidant defense has been shown via *in vivo* experiments on rats that have been put on a selenium-deficient-diet. Rats under oxidative stress due to selenium-deficiency display hepatic necrosis owing to selenium-deficiency associated with the loss of Gpx activity while selenium supplementation prevents this disease condition (Schwarz and Foltz, 1999).

Chronic selenium-deficiency may also occur in individuals with malabsorption and long term selenium-deficient parenteral nutrition. Selenium-deficiency causes myopathy as a result of the depletion of selenium-associated enzymes which protect cell membranes from damage by free radicals (See Diplock, 1994; See Rayman 2000).

1.11.2 Selenium-Deficiency and Alcoholism

One of the etiologies of HCC development is alcohol abuse. Alcohol is a source of oxidative stress. In alcohol-related liver damage, there is consistent evidence of enhanced production of free radicals and significant decrease of antioxidant defense. Alcohol-exposed cells are selectively depleted of GSH in mitochondria due to a defective functioning of the carrier responsible for the transport of GSH from cytosol into the mitochondrial matrix. This impaired transport sensitizes

hepatocytes to the prooxidant effects of cytokines and prooxidants generated by the oxidative metabolism of ethanol (Fernandez-Checa *et al.*, 1998). In the absence of GSH, GSH- and selenium-dependent antioxidant enzymes, such as Gpxs, cannot function.

Previously, it has been documented that in non-cirrhotic and cirrhotic alcoholics, serum levels of selenium were decreased by 17 and 48%, respectively, as compared to healthy controls. Plasma selenium levels in non-cirrhotic alcoholics who stopped drinking for two weeks and in non-alcoholic volunteers who had acute alcohol intake, did not change. In patients with primary biliary cirrhosis, the serum concentration of selenium was similar to that in the alcoholic cirrhotics (Valimaki *et al.*, 1983).

Another study demonstrated the significantly lower serum selenium concentrations of patients with liver cirrhosis and in those with hepatocellular carcinoma (Buljevac *et al.*, 1996). There are also studies correlating intrahepatic cholestasis to selenium-deficiency (Reyes, 1997). Recently, significantly reduced serum selenium levels were reported in a group of patients experiencing alcoholic cirrhosis (Guarini *et al.*, 1998).

In conclusion, the association of alcohol consumption, liver diseases and the decrease in serum selenium levels correlates with the increase in antioxidant status due to alcohol consumption.

1.11.3 Selenium-Deficiency and Viral Liver Diseases

To date, there are several reports implying the protective effect of selenium supplementation on virus induced liver damage. The protective effect of dietary selenium supplement has been reported to reduce HBV infection and liver cancer cases, by 77,2% and 75,8%, respectively, in animal studies performed with ducks (Yu *et al.*, 1997). Furthermore, preventive effect of dietary selenium supplementation on both HBV infection and liver cancer has been observed in a study performed on 130,471 individuals in Qidong. In this study, there was no incidence of HCC development among individuals who were positive for hepatitis

B surface antigen (HbsAg) supplemented with selenium, but upon termination of treatment, HCC developed at the same rate as control group, illustrating that a continuous selenium intake is crucial to maintain its chemopreventive effect (Yu *et al.*, 1997).

Recently, significantly reduced serum selenium levels were reported in a group of patients experiencing post-viral cirrhosis (Guarini *et al.*, 1998). Also, it has been stated that patients with HCV-related chronic liver disease have a significant decrease of blood levels of selenium (Loguercio *et al.*, 1997). In addition, the association between plasma selenium levels and risk of hepatocellular carcinoma among chronic carriers of HBV and/or HCV has been supported by a research performed among 7,342 men in Taiwan. The comparison was between the affected individuals who were positive for HBsAg and/or antibodies against hepatitis C virus (mostly HBsAg positive) and the healthy controls that were HbsAg-positive. Mean plasma selenium levels were significantly lower in the HCC cases than in the HBsAg-positive controls, indicating an inverse correlation between selenium levels and HCC (Yu *et al.*, 1999).

Another recent paper described the decrease in plasma selenium concentration in patients with post-viral cirrhosis. In parallel, increase in Gpx, and GST activity has been observed among these patients with high alanine aminotransferase (AIAT), which reveals liver damage. On the other hand, patients with low AIAT had low Gpx activity. Impaired intestinal absorption and binding of selenium by plasma proteins have been suggested as possible mechanisms of reduced selenium concentration. Increased formation of ROS or release of these enzymes from injured hepatocytes to plasma may cause changes in the activities of glutathione-dependent enzymes in plasma in these patients (Czuczejko *et al.*, 2002).

1.12 Selenium and HCC

The involvement of selenium-deficiency in the occurrence of liver cancer has been extensively pointed out, but, the molecular mechanisms underlying this implication are not known. The significance of selenium in human health, its

deficiency in HCC patients, and the key role it has in antioxidant mechanisms have been discussed in previous sections.

So far, reports demonstrating both the apoptosis-inducing and apoptosis-preventing effects of selenium in HCC cell lines exist. In fact, apoptosis due to selenium-deficiency-associated oxidative stress has been poorly characterized in selenium-deficient Gpx1 -/- mice (Cheng *et al.*, 2003). On the contrary, high doses of sodium selenite (Na_2SeO_3) induced oxidative stress and apoptosis in HepG2 cells. In this study, concurrent decline of intracellular GSH and increase of GSSH contents were observed in selenium treated cells (Shen *et al.*, 1999).

Protection conferred by selenium-deficiency against hepatocarcinogen AFB1 in the rat liver is associated with the increased hepatic expression of a GSTα5 (Glutathione S transferase alpha 5) that metabolize this mycotoxin. GSTα5 was found to be expressed throughout the centrilobular and midzonal areas of the liver lobule but was essentially absent from periportal hepatocytes (McLeod *et al.*, 1997). Besides, an inhibitory effect of selenium on the initiation and promotion stages of AFB1-induced preneoplastic foci and nodules in rat livers has been illustrated (Lei *et al.*, 1990).

1.13 Oxidative Stress and HCC

Recent studies have identified oxidative stress as an increased risk factor for the development of liver cancer.

Patients with hemochromatosis have 200 times more increased risk of HCC development as well as cirrhosis and liver failure compared with the normal population (Niederau *et al.*, 1985). Evidence of oxidative stress and subsequent generation of reactive aldehydes have been reported in hemochromatosis, and Wilson disease patients related to iron and copper overload, respectively. Membrane-dependent functions of mitochondria (oxidative metabolism) and lysosomes (membrane integrity, fluidity, pH) are damaged due to lipid peroxidation in the liver. It has also been demonstrated that metal overload induces DNA damage in the liver which might be a reason for malignant

transformation. Moreover, in the livers of rats with excess iron or copper, the levels of some antioxidants have been reported to decrease, which can be an indication of disturbance of intracellular pro-oxidant anti-oxidant balance in the liver (Britton, 1996).

Exposure of cultured rat hepatocytes to AFB1 has been demonstrated to cause generation of MDA, indication for lipid peroxidation, and lactate dehydrogenase, indication for cell injury (Shen *et al.*, 1995). It has been stated that AFB1 exposure considerably enhanced the occurrence of intracellular ROS (Shen *et al.*, 1996).

So far, the relation between viral infection, oxidative stress, and HCC has been illustrated. Mitochondrial targeted HbxAg of HBV has been reported to induce ROS generation and subsequently to activate certain transcription factors such as NFκB and STAT-3 (Waris *et al.*, 2001). A current research shows that patients with chronic HCV, chronic HBV, alcoholic hepatitis, and hepatocellular carcinoma have higher ROS levels than the controls (Valgimigli *et al.*, 2002). Moreover, HCV core protein expression increased lipid peroxidation products both *in vitro* and *in vivo* (Okuda *et al.*, 2002). Another study shows that lipid and protein oxidation occur in chronic hepatitis C patients, and causes oxidative damage in these patients (De Maria *et al.*, 1996). Another report supports De Maria *et al.*, by showing the elevation in lipid peroxidation and the ratio of oxidized to reduced glutathione in chronic hepatitis C patients (Jain *et al.*, 2002). In addition to all above, it is known that patients suffering from hepatitis C display an increased production of tumor necrosis factor-alpha (TNF-α), which is a cytokine that stimulates the generation of ROS (Larrea *et al.*, 1998).

All these data imply the physiological significance of HBV and HCV infection related to oxidative stress that can contribute to the liver disease pathogenesis associated with the infection of HBV and HCV.

1.14 Hepatocellular Carcinoma

HCC is the primary malignant tumor of the liver, ranking the fifth in men, and the eighth in women among other malignant tumors (Bosch *et al.*, 1999; Tabor, 2001). Estimated mortality from HCC was 564,000 per year in 2000 (Parkin *et al.*, 2003). Depending on the geographical region, the frequency of the incidence of HCC varies, eastern and southeastern Asia, some of the Western Pacific Islands, and most parts of sub-Saharan Africa being the regions with high incidence (Parkin *et al.*, 1992; Bosch *et al.*, 1999; Parkin *et al.*1999).

Adult hepatocytes are nondividing cells under normal physiological conditions. In fact, in response to a minor cell loss consequent to aging or apoptosis, a small number of hepatocytes go through cell division. On the other hand, following exposure to chemicals (i.e., alcohol, AFB1), viral (i.e., HBV, HCV), and cellular factors (i.e., ROS, excess iron and copper in liver) and during liver injury considerable cell death can occur. In response to this, to compensate the liver loss, proliferating fraction of hepatocytes increases. Notably, chronic exposure to such factors that lead to liver injury provokes liver regeneration. Chronic liver regeneration may itself be a source of spontaneous gene mutations (See Öztürk, 1999).

The development of hepatocellular carcinogenesis requires accumulation of both epigenetic and irreversible structural genomic alterations. This is a slow process that can take 10-30 years and first gives rise to phenotypically altered hepatocytes that are at the stage of chronic hepatitis and/or cirrhosis. Then, these hepatocytes go through a sequential process of preneoplastic stage that includes dysplasia, and finally to neoplastic stage in which irreversible genetic aberrations are gathered in the hepatocytes (Thorgeirsson and Grisham, 2002).

1.14.1 Viral Factors

Epidemiological, clinical and experimental studies link persistent HBV infection to HCC. Cellular composition of HBV infected hepatocytes is altered consequent to hepatocyte necrosis (Rogler *et al.*, 1987), inflammation (infiltrating

hematopoietic cells) and regeneration (Robinson *et al.*, 1990). Liver injury due to viral exposure provokes an increase in proliferating hepatocytes. Chronic state of cell death and regeneration may be the source of genetic errors in hepatocarcinogenesis (Öztürk, 1994; Öztürk, 1999).

It is documented that replicating HBV has no oncogenic impact on hepatocytes. Integration can end up with the alteration in the expression pattern of the host genes (Öztürk and Çetin-Atalay, 2002). In HCC, HBV was shown to integrate into the cyclin A gene (Wang *et al.*, 1992), which is required for cellular proliferation, into the retinoic acid receptor gene (Dejean and de The, 1990), which is involved in cellular differentiation, and mevalonate kinase gene (Graef *et al.*, 1994), which is indirectly involved in farnesylation of growth related proteins like ras oncoprotein.

In most of the tumor cells, HBV genome persists. HBV encoded X antigen (HbxAg), and PreS2 structural proteins encoded by viral S gene, are maintained and these are transactivator proteins (Henkler and Koshy, 1996). In humans, HbxAg and PreS2 proteins of the virus transactivate cellular genes. For example, AP-1 and NFκB transcription factors are activated by these viral proteins which, in turn, lead to the activation of signalling pathways. By this way genes for proliferation are controlled (Benn and Schneider, 1994; Hildt *et al.*, 2002).

Moreover, HbxAg has been documented to associate with p53 tumor suppressor protein both i*n vitro* and *in vivo* (see Wang *et al.*, 2002) and it is also involved in the regulation of expression of TGF-β1, insulin like growth factor I receptor (IGF1R), and insulin like growth factor-II (IGF2) genes (Yoo *et al.*, 1996; Kang-Park *et al.*, 2001). Still, the exact mechanism how HbxAg contributes to the development of malignant hepatocytes remains unknown.

It is presently unclear how HCV, which is an RNA virus, establishes a persistent infection in hepatocytes. There is no *in situ* evidence of reverse transcription DNA intermediates implicating that HCV cannot integrate into host (Itoh *et al*., 1986; Fong *et al.,* 1991). Some variants of HCV have been shown to replicate in both malignant and nonmalignant tissues, others replicate only in HCC tissue (Gerber

et al., 1992; Takeda *et al*., 1992; Niu *et al*., 1995). HCV may contribute to hepatocarcinogenesis as a consequence of its central role in the appearance and progression of necroinflammatory liver disease. Continuous cell death followed by regeneration may lead to accumulation of genetic damage in the infected hepatocytes. There is also increasing evidence for a direct contribution of several HCV gene products to the development of the transformed phenotype, although none of the putative mechanisms involved in tumor formation have been strongly supported by *in vivo* evidence (Moriya *et al.*, 1998; Öztürk and Çetin-Atalay, 2002).

Direct role of the core protein in HCC development was demonstrated experimentally using mice transgenic for HCV core protein (Moriya *et al.*, 1998). Among the examples of the action of core protein, down-regulation of p21 (WAF1) expression (Dubourdeau *et al.*, 2002), activation of the Ras/Raf signaling pathway and anti-apoptotic behavior of the cells (Shimotohno *et al.*, 2002) can be counted. Another region that confers oncogenic potential to the virus is the 5' end of the HCV genome that encodes nonstructural protein 3 (NS5A) and transforms cells *in vitro*. NS5A has been shown to associate physically and functionally with p53 *in vivo* (Qadri *et al.*, 2002).

1.14.2 Dietary Factors

International Agency for Cancer Research (IARC), like HBV and HCV infection, considered exposure to naturally occurring aflotoxins as carcinogenic to humans (Ozturk and Cetin-Atalay, 2002). Among the aflatoxins, AFB1 is the main form present in contaminated foods. During oxidative metabolism of AFB1 the first derivative of AFB1 formed, has a tendency towards the nucleophilic centers of DNA, RNA and proteins. Transversions and transitions in AFB1 induced HCC in experimental animals was reported. Patients who are at high risk of exposure to AFB1 were described to have a hotspot codon 249 mutation (AG*G* to AG*T*) in tumor suppressor p53 gene (Bressac *et al.*, 1991; Hsu *et al.*, 1991).

Chronic alcohol abuse is a tumorigenic risk factor for many organs. There is evidence that cirrhosis is the dose dependent effect of alcohol consumption and

the source for ethanol-associated liver cancer (Ohnishi *et al.*, 1982). In addition to alcohol, iron overload (Win *et al.*, 2000), and copper overload (Haratake *et al.*, 1987) are potential promoters of the development of HCC.

1.14.3 Genetic Alterations

The order of events that end up with full malignancy of hepatocytes is most probably the genomic instability, insensitivity to antigrowth signals, self-sufficiency in growth signals, ability to escape apoptosis, unrestricted replicative potential, sustained angiogenesis, and tissue invasion and metastasis as summarized in Figure 1.12.1 (Öztürk and Çetin-Atalay, 2002).

In HCC, chromosomal gain was observed on chromosomes 1q, 8q, and 7q, where possible ongogenes might be located, while gene dose loss was observed on 1p, 4q, 8p, 9p, 13q, 16p, 16q, and 17p, where possible tumor suppressor genes might be located (Pineau *et al.,* 1999; Qin *et al.*, 1999; Yakicier *et al.*, 2001; Zhao *et al.*, 2001). Frequent allelic imbalance, and loss of heterozygosity (LOH), were observed on chromosomes 1p, 4q, 6p, 8p, 13q, 16q, and 17p. LOH on chromosomes 17p, 13q, 9p, 6q, and 16p can be linked to the inactivation of p53, retinoblastoma (pRB), p16, IGF2R, and axin genes, respectively.

Wnt/β-catenin signal transduction is involved in tumorigenesis of many tissues, including liver. β-catenin (Nhieu *et al.*, 1999; Wong *et al.*, 2001) and axin (Satoh *et al.*, 2000) mutations can be observed in HCC cases. On the other hand, somatic adenomatous polyposis coli (APC) and E-cadherin gene mutations are very rare in HCC (Tannapfel and Wittekind *et al.*, 2002).

TGF-β is an apoptotic and growth inhibitor protein for hepatocytes. Sma and mad related 2 (Smad2) and Sma and mad related 4 (Smad4) (Yakicier *et al.*, 1999), TGF-β receptor II (TGF-βR1RII; Kawate *et al.*, 1999; Enomoto *et al.*, 2001), M6P/IGF2R, and PTEN/MMAC1/TEP1 gene mutations are documented in this pathway (De Souza et al., 1995; Yao *et al.*, 1999).

p53 is a tumor suppressor gene involved in cell cycle arrest, apoptosis, and senescence and it is activated following DNA-damage, oxidative stress, decreased nucleotide reserve, and oncogenic activation (Blagosklonny, 2002; Amundson *et al.*, 1998; Atadja *et al.*, 1995; Itahana *et al.*, 2001). In HCC, 30% of the cases harbor mutations of the p53 gene, all of them being somatic mutations. P53 *codon 249 mutation (*AG*G to* AG*T* transversion resulting in arginine to serine*)* is a specific hotspot mutation in HCC observed in individuals who are at high risk for AFB1 exposure together with HBV infection (Bressac *et al*., 1991; Hsu *et al.*, 1991). Specific mutations in neither p73 nor p63 have been identified until now. The overexpression of p73, which is a p53 homologue, has been demonstrated in a subset of HCC cases (Tannapfel *et al.*, 1999), and the upregulated form was shown to be the trascriptionally active form of p73 in HCC (Sayan *et al.*, 2001a).

Many of the gene alterations observed in HCC are related to cell cycle regulatory functions of pRb protein. Cyclin D amplification or loss of p16 protein function leads to constitutive phosphorylation of pRb protein triggering cell cycle through G_1-S phase by the release of a class of transcription factors called E2Fs from pRb. In order to keep the cell cycle under control upon pRb phosphorylation, p14 (ARF: alternative reading frame), senses the mitogenic signals. Subsequently, p14 prompts p53 dependent cell cycle arrest or apoptosis, through inhibiting mouse double minute 2 (Mdm2), which is a negative regulator of p53, by triggering p53-dependent transcriptional response (Tannapfel and Wittekind, 2002).

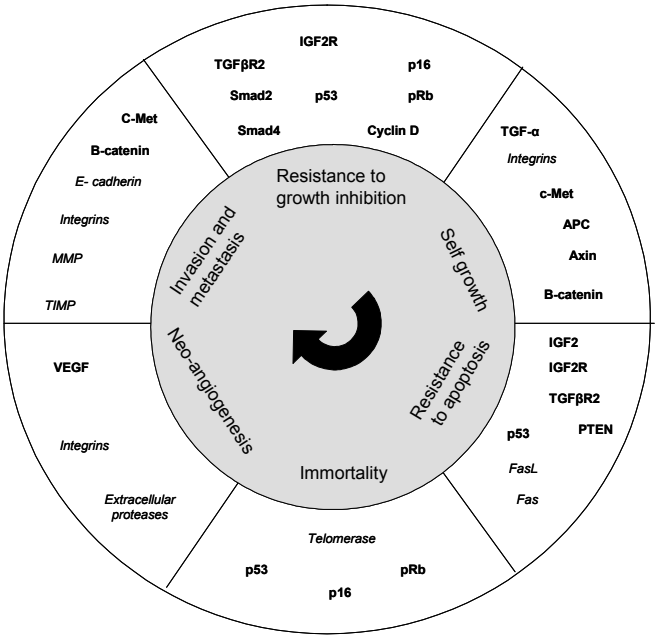

Figure 1.14.1: Molecules Targeted in HCC. Acquired phenotypic features of hepatocellular carcinoma and molecules in different stages of hepatocellular carcinoma development. Genes known to display structural alteration are in bold whereas those with altered expression are in italic (See Öztürk and Çetin-Atalay, 2002).

CHAPTER 2. AIM and STRATEGY

2.1 Experimental Introduction of *in vitro* Selenium-Deficiency

With the intention of observing the *in vitro* response of hepatocellular carcinoma cell lines to selenium-deficiency, and consequently, understanding the significance of selenium-deficiency in tumoregenesis of hepatocytes, Huh-7 cells were maintained under selenium-adequate DMEM (0.1% FBS and 100 nM Na_2SeO_3) and selenium-deficient DMEM (standard culture medium supplemented with 0.1% FBS). Firstly, *in vitro* response of Huh-7 cells to selenium-deficiency was assessed by checking cellular Gpx activity of these cells in DMEM via an *in situ* staining technique (Sun *et al.*, 1988). As expected, Huh-7 cells responded to selenium-deficiency with a total loss of Gpx activity at 96^{th} hour since there was no selenium availibility. On the contrary, they displayed Gpx activity when maintained in DMEM under selenium-adequate conditions as shown in Figure 2.1.1 (İnce, 1999).

In order to see the morphological changes in Huh-7 cells, they were plated in selenium-adequate or selenium-deficient medium. The cells were analyzed daily under the light microscopy. On the 3^{rd} day of the culture, some morphological changes were observed under selenium-deficient experimental conditions, and on the 4^{th} day, the number of cells with morphological changes increased. These changes were not present under selenium-adequate conditions. Figure 2.1.2 shows the apparent morphological changes of Huh-7 cells under selenium-deficiency. Cells were growing slowly under both conditions, most probably due to the low levels of serum (İnce, 1999).

Huh-7

Figure 2.1.1: *In vitro* response of Huh-7 cells to selenium-deficiency. Huh-7 cells were grown in selenium supplemented (Se+) or selenium-deficient (Se−) conditions. *In vitro* selenium-deficiency was tested by the loss of Gpx activity. At 96th hour, cells were solubilized as described İnce, 1999. 50 µg of soluble proteins from cell lysates were separated by native polyacrylamide gel electrophoresis, followed by *in situ* staining for Gpx with a colorimetric assay. Mouse liver tissue lysate was used as a positive control.

Huh-7

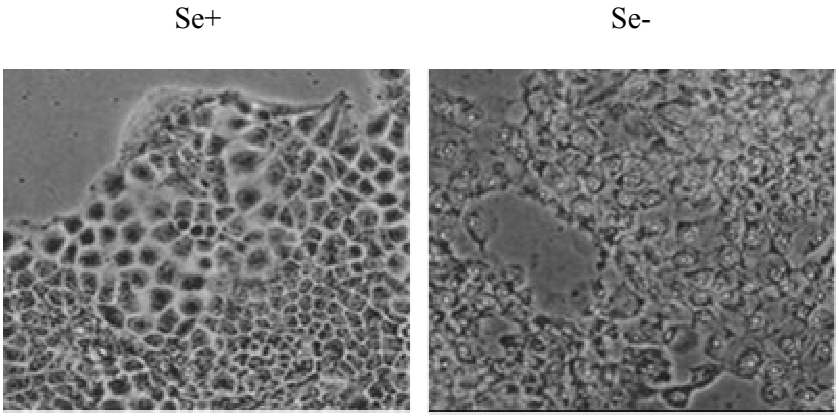

Figure 2.1.2: Death of Huh-7 cells under selenium-deficiency in DMEM. Huh-7 cells were examined under inverted light microscopy on a daily basis, and photographs of Huh-7 cells under selenium-adequate (Se+) and selenium-deficient (Se-) conditions were taken at day 4. Note the morphological changes of Huh-7 cells under selenium-deficient conditions but not under selenium-adequate conditions. Original magnification 100×.

Next, the nature of the cell death exhibited by Huh-7 cells under selenium-deficiency was characterized. Huh-7 cells could enter apoptosis as a result of oxidative stress under selenium-deficiency due to the loss of Gpx activity. The relation between oxidative stress and apoptosis has been well documented in the literature (Chandra *et al.*, 2000); nevertheless, apoptosis due to selenium-deficiency associated with oxidative stress has been poorly characterized (Cheng *et al.*, 2003). Cellular apoptotic machinery provoked under our oxidative stress conditions could be a mechanism that HCC-derived Huh-7 cells were using to avoid proliferation under oxidative stress and to prevent malignancy.

Apoptotic cells exhibit extensive DNA cleavage usually during early stages of this controlled cell death mechanism. Cleavage may produce double-stranded, low molecular weight DNA fragments as well as single-stranded high molecular weight DNA fragments. It is possible to detect those strand breaks by enzymatic end labeling of free 3'OH termini with modified nucleotides with an assay called TUNEL. It is specific to apoptotic strand breaks and direct labeling with fluorescein-dUTP allows detecting the apoptotic cells under fluorescence microscopy (http://www.roche-applied-science.com).

In order to clarify the type of cell death mechanism, TUNEL assay was performed as shown in Figure 2.1.3. Huh-7 cells were grown in selenium-adequate and selenium-deficient DMEM medium for 4 days. Under selenium-deficient conditions Huh-7 cells had condensed nuclei, as shown by Hoechst 33258. Those cells with condensed nuclei were positive for TUNEL assay with green fluorescence. This observation demonstrated that those cells displayed the type of cell death, specific to apoptosis. On the contrary, Huh-7 cells under selenium-adequate conditions had neither condensed nuclei nor positivity for TUNEL assay (İnce, 1999).

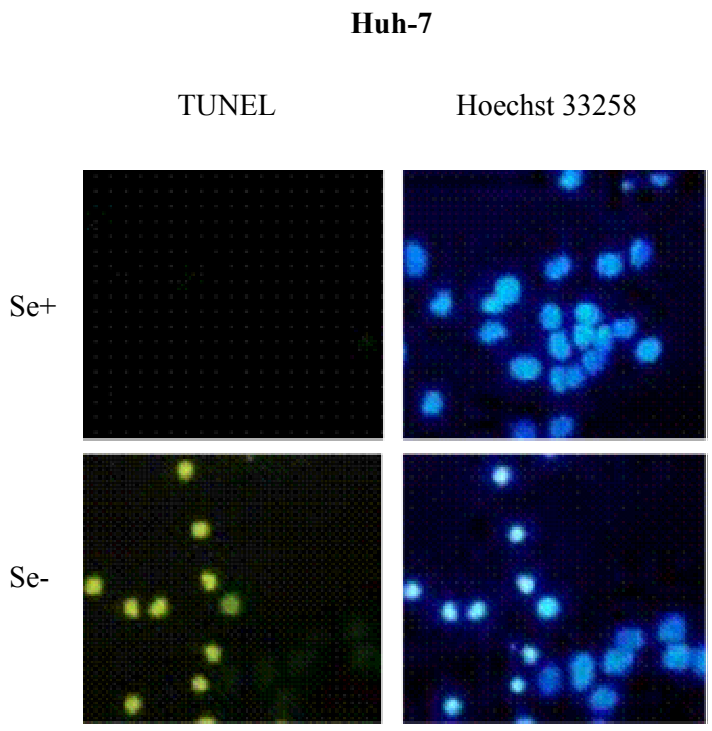

Figure 2.1.3: TUNEL assay of Huh-7 cells cultured in DMEM. Cells were cultured in selenium-adequate (Se+) and selenium-deficient (Se-) DMEM. Left panel shows TUNEL staining and right panel shows the nuclear Hoechst 33258 staining of the corresponding area of TUNEL staining. Note the TUNEL positive signals of Huh-7 cells with condensed nuclei under selenium-deficient conditions.

Asymmetric distribution of phospholipids, phosphotidiylserine residues being on the inner membrane and the neutral phospholipids being mostly on the outer membrane, is totally lost in theearly stages of apoptosis. The amount of anionic phosphotidiylserine residues on the outer surface increases, and the residues are exposed to extracellular fluid (Vermes *et al.*, 1995). Annexin V is known to be a calcium-dependent phospholipid-binding protein with high affinity to phosphotidiylserine residues. Thus, Annexin V binds to the phosphotidiylserine residues on the outer membrane of apoptotic cells, being a good marker to detect early apoptotic cells (Homburg *et al.*, 1995; Verhoven *et al.*, 1995).

In order to verify apoptotic kind of cell death Annexin-V assay was performed as shown in Figure 2.1.4. Huh-7 cells were grown in selenium-adequate and

selenium-deficient DMEM medium for 4 days. Under selenium-deficient conditions Huh-7 cells had condensed nuclei, as shown by Hoechst 33258 staining. Those cells with condensed nuclei were positive also for Annexin V staining. So, it was verified that Huh-7 cells die by apoptosis under selenium-deficiency. On the contrary, Huh-7 cells under selenium-adequate conditions had neither condensed nuclei nor positivity for Annexin V assay like in the case of TUNEL assay (İnce, 1999).

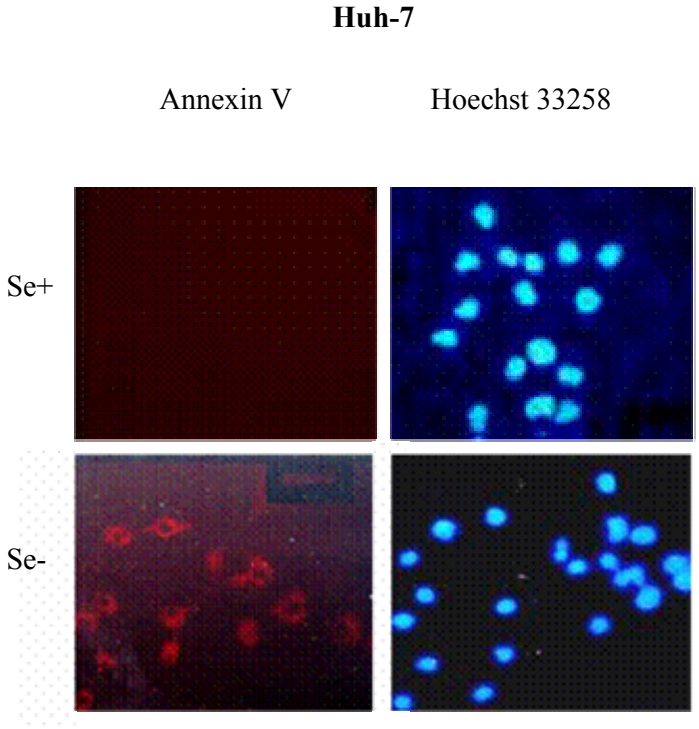

Figure 2.1.4: Annexin V assay of Huh-7 cells cultured in DMEM. Cells were cultured in selenium-adequate (Se+) and selenium-deficient (Se-) DMEM. Left panel shows Annexin V staining and right panel shows the nuclear Hoechst 33258 staining of the corresponding area of Annexin staining. Note the Annexin positive signals of Huh-7 cells with condensed nuclei under selenium-deficient conditions (lower panel). Huh-7 cells under selenium-adequate conditions do not display any Annexin positive signals (upper panel).

Following this, Hep3B-TR cells (TGF-β resistant-subclone of Hep3B; Hasegawa *et al.*, 1995) were tested in the same manner for Gpx activity as shown in Figure 2.1.5. Hep3B-TR cells had Gpx activity under selenium-adequate conditions, but not under selenium-deficient conditions like Huh-7 cells, as expected.

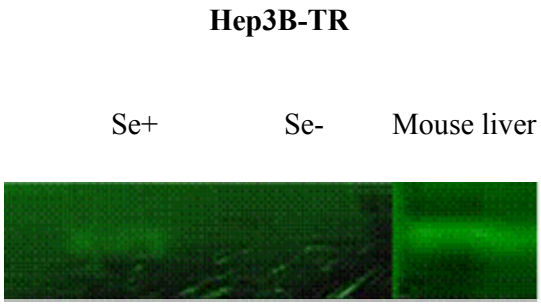

Figure 2.1.5: *In vitro* response of Hep3B-TR cells to selenium-deficiency. Hep3B-TR cells were grown in selenium-supplemented (Se+) or selenium-deficient (Se−) conditions. *in vitro* selenium-deficiency was tested by the loss of Gpx activity. At 96th hour, cells were solubilized as described in İnce, 1999 and 50 µg of soluble proteins from cell lysates were resolved by native polyacrylamide gel electrophoresis, followed by *in situ* staining for Gpx with a colorimetric assay. Mouse liver tissue lysate was used as a positive control

In order to see the morphological changes in Hep3B-TR cells, cells were plated under selenium-adequate and selenium-deficient conditions in DMEM. The cells were analyzed daily under the light microscopy. The morphology of the cells under both conditions was similar, with no visible difference under light microscopy as shown in Figure 2.1.6. Like in the case of Huh-7 cells, Hep3B-TR cells were growing slowly, most probably due to the absence of serum, and consequently growth hormones necessary for proliferation.

Hep3B-TR cells were expected to be negative for both TUNEL and Annexin V, assays since they were healthy looking on the 4th day of the culture. To confirm this, TUNEL and Annexin V assays were performed and are shown in Figure

2.1.7 and Figure 2.1.8, respectively. Hep3B-TR cells were negative for the assays, indicating that they were non-apoptotic.

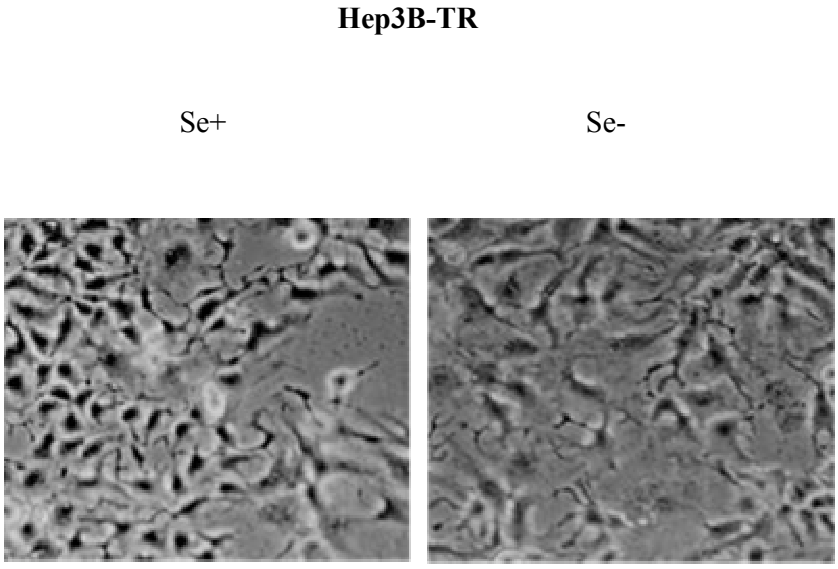

Figure 2.1.6: Survival of Hep3B-TR cells under selenium-deficiency. Hep3B-TR cells are examined under inverted light microscopy on a daily basis, and photographs of Hep3B-TR cells under selenium-adequate (Se+) or selenium-deficient (Se-) conditions were taken at day 4. Hep3B-TR cells did not show any morphological change under selenium-deficient conditions. Original magnification 100×.

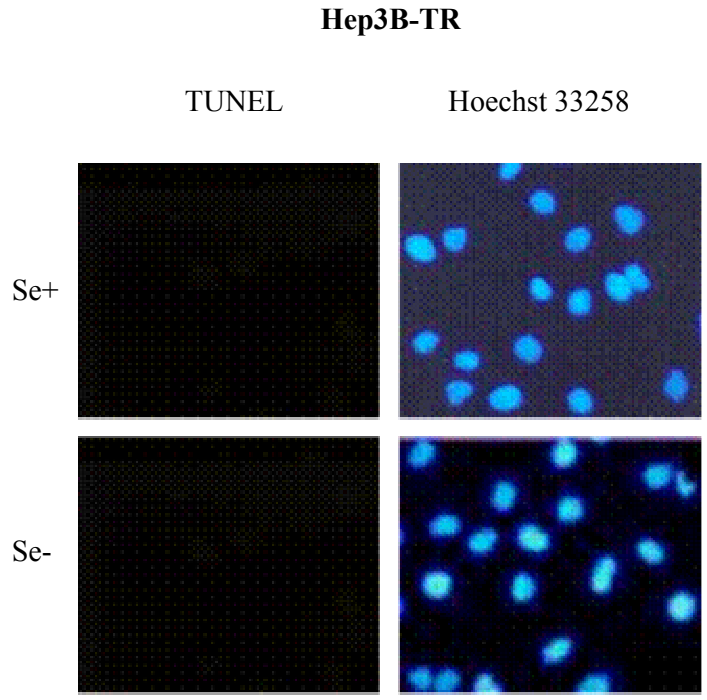

Figure 2.1.7: TUNEL assay of Hep3B-TR cells cultured in DMEM. Cells were cultured in selenium-adequate (Se+) and selenium-deficient (Se-) DMEM. Left panel shows TUNEL staining and right panel shows nuclear Hoechst 33258 staining of the corresponding area of TUNEL staining. Note that Hep3B-TR cells were negative for TUNEL assay under both conditions.

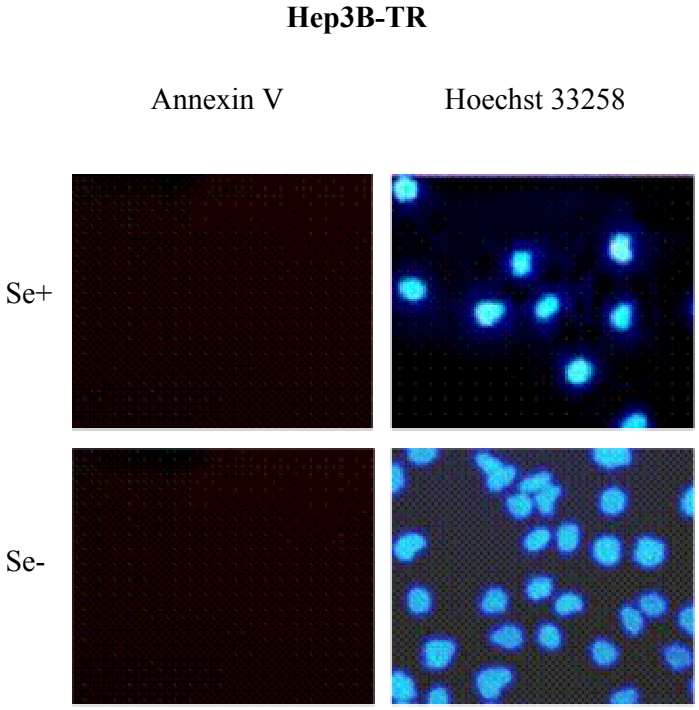

Figure 2.1.8: Annexin V assay of Hep3B-TR cells cultured in DMEM. Cells were cultured in selenium-adequate (Se+) and selenium-deficient (Se-) DMEM. Left panel shows Annexin V staining and right panel shows the nuclear Hoechst 33258 staining of the corresponding area of Annexin V staining. Note that Hep3B-TR cells were negative for Annexin V assay under both conditions.

2.2 Aim

In vivo hepatic response to selenium-deficiency has not been characterized as apoptotic cell death. It is well documented that selenium-deficiency causes oxidative stress and apoptosis is a common cellular response to oxidative stress. In section 2.1, it is shown that Huh-7 and Hep3B-TR cells respond to selenium-deficiency with a total loss of Gpx activity and the activity is recovered under selenium-adequate conditions. In addition, Huh-7 cells die by apoptosis under selenium-deficiency, while Hep3B-TR cells do not.

The association of selenium-deficiency with hepatic disease conditions including hepatocellular carcinoma is well documented. Yet, the underlying molecular mechanisms of this association are poorly characterized. In order to improve and add to the available data in the literature related to selenium-deficiency and development of hepatocellular carcinogenesis, and finally to discover the phenomena lying under the malignant type of response of hepatocytes to selenium-deficient conditions, we developed an *in vitro* experimental model using HCC-derived human cell lines.

2.3 Strategy

We decided to find out the reason of differential response of Hep3B-TR cells to selenium-deficiency. It could either be due to the resistancy to selenium-deficiency induced oxidative stress or selenium-deficiency induced apoptosis under these conditions. Meanwhile, we wanted to see whether the response of HCC cell lines is affected by inclusion of Copper/Zinc ions (by using HAM's medium that contains copper and zinc) and Vitamin E, which have antioxidant roles. As it was mentioned in Chapter 1, Copper/Zinc is important for the function of SOD enzyme found upstream of Gpx. Vitamin E has a protective scavenger effect against free radical damage downstream of Gpx. Next, we wanted to generalize the results for the development of HCC by using 11 more cell lines.

Finally, we investigated the response of cell lines of other cancer tissues, such as breast, colon, and melanoma, to selenium-deficiency induced stress conditions to

have an overall idea about the effects of selenium-deficiency in other tumor cell lines.

CHAPTER 3. MATERIALS and METHODS

3.1 Materials

3.1.1 General Reagents

All laboratory chemicals were analytical grade from Sigma Biosciences Chemical Company Ltd., St. Louis, MO, U.S.A, Farmitalia Carlo Erba, Milano, Italy, Merck (Schucdarf, Germany), and Riedel-de Haën, Deisenhofen, Germany.

3.1.2 Tissue Culture Reagents

Dulbecco's Modified Eagle's Medium (DMEM), HAM's medium (with 10 μM Copper and 3mM Zinc), nonessential amino acids, penicillin/streptomycin, trypsin /EDTA, and fetal bovine serum (FBS) were purchased from BioChrom, Berlin, Germany. Na_2SeO_3 was from Sigma, Taufkircher, Germany, and Vitamin E (D-α-tocopherol, Grandpherol®) was from İlsan, İltaş A.Ş., Turkey, pure Vitamin E was from BASF, Germany, geneticin (G418 sulfate) was from GibcoBRL, Life tech., U.S.A. Tissue culture petri dishes, 15 and 50 ml polycarbonate centrifuge tubes with lids and cryotubes were from Costar Corp., Cambridge, England.

3.1.3 Oxidative Stress Detection Reagents

2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was from Sigma, Taufkircher, Germany.

3.1.4 Apoptosis Assay Reagents

Annexin V-PE reagent was from PharMingen, BD, USA. Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling (TUNEL) In Situ Cell Death Detection Kit was from Roche Mannheim, Germany. Monoclonal antibodies raised against NAPO (Negative in Apoptosis), which is a nuclear antigen, were produced in house (See Sayan *et al.*, 2001).

3.1.5 Immunofluorescence Reagents

Paraformaldehyde, Bovine Serum Albumine (BSA), and Hoechst 33258 were purchased from Sigma, Taufkircher, Germany, Tween-20 was from Amresco, Ohio, USA. Mouse anti-cytochrome c monoclonal antibody was purchased from Pharmingen, BD, USA, and fluorescein isothiocyanate (FITC) conjugated secondary antibody, goat anti-mouse immunoglobulins-F(ab')2 was purchased from DAKO, CA, USA

3.1.6 Western Blot Reagents

Rabbit anti-Akt polyclonal antibody, and mouse anti-poly[ADP-ribose] polymerase (PARP) monoclonal antibody were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. PVDF (polyvinilydene difluoride) membrane was from Roche, Mannheim, Germany, nitrocellulose western blotting membrane was from Sartorious, Goettingen, Germany, and negative X-ray film was from Kodak, France. Horse Radish Peroxidase (HRP) conjugated secondary antibody was from DAKO, CA, USA. Enhanced ChemiLuminescence (ECL) plus detection kit was from Amersham-Pharmacia, Buckinghamshire, England. D. Bellet, France, kindly provided mouse anti-cytokeratin 18/JAR13 (CK18) monoclonal antibody.

3.1.7 Cell Lines

12 hepetocellular carcinoma derived, namely, Huh-7, Hep40, Hep3B, Hep3B-TR, FOCUS, SNU 475, SNU 449, SNU 423, SNU 387, SNU 182, PLC/PRF/5, and

Mahlavu, 1 hepatoblastoma derived cell line, namely, HepG2 (Park *et al.*, 1995; Simon *et al.*, 1995) and 1 HBV-transfected 2.2.15 clone of the HepG2 cell line that produces infectious HBV virions (Sells *et al.*, 1987) were used in this project.

3.1.8 Polymerase Chain Reaction Reagents

Taq polymerase, dNTP mix, and $MgCl_2$ were from MBI Fermentas, Germany. Primers used were synthesized by İONTEK (Bursa, Turkey). Sequence of the primers used is given in section 3.4.8 in Table 3.4.8.1.

3.2 Solutions and Media

3.2.1 Tissue Culture Reagents

Standard medium	2 mM L-Glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin in DMEM or HAM's medium.
Control Medium	Standard DMEM supplemented with 10% FBS.
Selenium-Adequate Medium	Standard medium with 0.01% FBS and 0.1 μM Na_2SeO_3.
Selenium-Deficient Medium	Standard medium with 0.01% FBS.
Freeze medium	65% DMEM control medium, 25% FBS, and 10% DMSO in 1 ml per cell line.
Geneticin	Stock solution of 500 mg/ml in ddH_2O, sterilized by filtration and

	stored at -20°C. Diluted to working concentration of 400 μg/ml.
Vitamin E	2 M Vitamin E is diluted to 50 μM in a final of 0.25% DMSO. Prepared freshly.
10 X PBS (phosphate buffered saline)	Stock solution of 1.37 M NaCl (80 g), 27 mM KCl (2 g), 43 mM Na_2HPO_4 (14.4 g), and 14 mM (2.4 g) KH_2PO_4 in 1 lt ddH_2O, pH 7.2. Diluted to working concentration of 1X.

3.2.2 Oxidative Stress Reagents

DCFH-DA Solution	10 mM HEPES (pH 7.5), 10 mM glucose, and 1 μM DCFH-DA (dissolved in methanol) in 1X PBS. Prepared freshly.

3.2.3 Apoptotic Assay Reagents

Annexin V Assay

Binding Buffer	10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$.

TUNEL Assay

In Situ Cell Death Detection Kit	Bottle 1 (enzyme solution) and bottle 2 (label solution). Store at -20°C.

TUNEL Reaction Mixture	50 µl from bottle 1 is added to 450 µl from bottle 2, mixed and kept on ice. Prepared freshly.
Permeabilization Solution	0.1% Triton-X-100, 0.1% sodium citrate.
4% Praformaldehyde	40 mg/ml paraformaldehyde in PBS, pH 7.4, and stored at 20°C.
0.075% Saponin	0.75 mg/ml saponin in PBS.

NAPO Assay

Permeabilization Buffer	3.4 ml sodium citrate, 100 µl Triton-X-100 in 100 ml PBS (0.1% Triton-X-100, 0.1% sodium citrate in PBS).

3.2.4 Immunofluorescence Reagents

PBS-T	0.1% Tween-20 in 1 X PBS.
Paraformaldehyde	4% paraformaldehyde (4 g in 100 ml) in PBS. Stir 4 hours at 58°C, aliquot and store at -20°C.
Hoechst 33258 fluorochromo dye	Stock solution of 300 µg/ml in ddH_2O. Diluted to working concentration of 3 µg/ml. Store at 4°C.
BSA	3% BSA in 1 X PBS (3 g in 100 ml).

Antibody Dilution	Antibody in 0.1% Tween-20 in 1 X PBS.

3.2.5 SDS PAGE Reagents

Lower Gel Buffer	250 mM Tris-base (pH 8.8), 0.2% SDS. Store at 4°C.
Upper Gel Buffer	750 mM Tris-base (pH 6.8), 0.2% SDS. Store at 4°C.
24% Acrylamide/Bisacrylamide mix	24 g acrylamide (24%), and 0.64 g bisacrylamide (0.64%) in 100 ml ddH_2O. Filter, and degas by vacuum. Store at 4°C in dark.
10% Ammonium persulfate (APS)	0.1 g APS in 1 ml (100 mg/ml) ddH_2O. Store at -20°C.

3.2.6 Western Blot Reagents

NP-40 (Nonidet-P40) Lysis Buffer	150 mM NaCl, 50 mM Tris-HCl (pH 8). Store at 4°C. Add 1% Igepal and 1% protease inhibitor freshly.
Bradford Stock Solution	100 ml 95% EtOH, 200 ml 88% phosphoric acid, 350 mg Serva Blue G. Store at 4°C in dark.
Bradford working solution	42.5 ml ddH_2O, 1.5 ml 95% EtOH, 3 ml 88% phosphoric acid, 3 ml Bradford stock solution. Store at room temperature in dark.

BSA Standard	1 mg/ml BSA in ddH_2O is stored at -20°C.
5 X Cracking Buffer	Stock solution of 125 mM Tris-base (pH 6.8), 2.5% SDS, 5 mM EDTA, 0.05% Bromophenolblue, 25% glycerol. 2-β-Mercaptanol added freshly and Diluted to working concentration of 1 X with the sample.
10 X Tris Buffer Saline (TBS)	100 mM Tris-base (12.1 g), 1.5 M NaCl (87.66), pH 7.5 in ddH_2O. Diluted to working concentration of 1 X. Stored at room temperature.
TBS-T	0.1% Tween-20 in TBS
Tris-Glycine Transfer Buffer	48 mM Tris-HCl (5.2 g), 39 mM glycine (2.93 g), 0.037% SDS (0.37 g or 3.75 ml from 10 % stock), 20% methanol (200 ml), pH 9.2, in 1 lt ddH_2O, (pH not adjusted). Store at 4°C.
Blocking Solution	5% (w/v) non-fat milk, 0.1% Tween-20 in TBS. (Prepared freshly).
Antibody Dilution	Antibody in 2.5% (w/v) non-fat milk, 0,1% Tween-20 in TBS. (Prepared freshly).

3.3 Equipment

Vertical minigel EC120	EC Apparatus Corporation
Power Supply	Bio-Rad, Power-pac200
Centrifuge GS-15R (rotors S4180 and F3602)	Beckmann
Transblot-Semi Dry	BioRad
Hyperprocessor	Amersham Life Science
CO_2 Incubator	Cell House, Holten
Laminar Flow Hood	Holten
Spectrophotometer DU 640	Beckmann
Inverted Light Microscope, Axiovert 25	Zeiss
Fluorescent Microscope Axioscope 50	Zeiss
Digital Photograph Machine	Kodak
GeneAmp System 9600	Perkin-Elmer

3.4 Methods

3.4.1 Tissue Culture

3.4.1.1 Growth Conditions

Cell lines were cultured at 37°C under 5% CO_2 in control medium unless otherwise specified. HepG2-2.2.15 cells were cultured at 37°C under 5% CO_2 in control medium with 400 μg/ml.

3.4.1.2 Thawing Cell Lines

A vial of cell line that was previously liquid nitrogen frozen was taken out of the liquid nitrogen tank, immediately put on ice and quickly thawed in water bath at 37°C. Thawed cells were gently resuspended with a pipette and were transferred to a 15 ml sterile tube that has 2-3 ml growth medium. Next, cells were centrifuged at 1400 rpm at 4°C for 5 minutes and the supernatant was carefully aspirated. Finally, the cell pellet was gently resuspended in 7 ml growth medium, transferred to 100 mm sterile dish, and incubated at 37°C overnight in a sterile humidified incubator provided with 5% CO_2. The following day, cells were checked under inverted light microscope. The attached cells that reached 70-80% confluency were subcultured and culture medium of less confluent cells was replaced with fresh medium after washing with PBS.

3.4.1.3 Sub-culture of Cell Lines

The culture medium of attached cells with 70-80% confluency was aspirated, cells were washed two times with PBS that was pre-warmed at 37°C, treated with 1 ml trypsin/EDTA, and incubated at 37°C for 3 minutes to be detached. In order to inactivate the trypsin, 5 ml growth medium that contains FBS was added on the cells. The cells were splitted in a dilution depending on the usage aim and the

growth characteristics of the cell line of interest and incubated at 37°C in a sterile humidified incubator provided with 5% CO_2.

3.4.1.4 Cryopreservation of Cell Lines

The medium of the cells that are 70-80% confluent in 150 mm was aspirated, the cells were washed two times with PBS that was pre-warmed at 37°C, treated with 1 ml trypsin/EDTA, and incubated at 37°C for 3 minutes to be detached. In order to inactivate the trypsin, 5 ml growth medium that contains FBS was added on the cells. The cells were counted and centrifuged at 1400 rpm at 4°C for 5 minutes, the cell pellet was resuspended in 1 ml freeze medium at a concentration of $4x10^6$cells/ml and placed into cryotubes. The tubes were kept at -80°C overnight and transferred to liquid nitrogen tank the next day.

3.4.1.5 Generation of *in vitro* Selenium-Adequate and Selenium-Deficient Medium

Serum-Free Selenium-Adequate Medium

A total of 41 cell lines were cultured at 37°C under 5% CO_2 in standard culture medium (DMEM or HAM's), supplemented with 0.01% FBS, and 0.1 μM Na_2SeO_3.

Serum-Free Selenium-Deficient Medium

A total of 14 HCC, 14 breast, 10 colon, and 3 melanoma cell lines were cultured at 37°C under 5% CO_2 in standard culture medium (DMEM or HAM's), supplemented with 0.01% FBS without any Na_2SeO_3.

3.4.1.6 Culture of HCC Cell Lines under Serum-Free Medium

HCC cell lines were cultured at 37°C under 5% CO_2 in control culture medium, overnight. 16 hours later mediums were aspirated, cells were washed two times

with pre-warmed PBS and the medium was replaced with either serum-free selenium-adequate or serum-free selenium-deficient medium and incubated for indicated times.

3.4.1.7 Treatment of HCC Cell Lines with H_2O_2

HCC cell lines were cultured at 37°C under 5% CO_2 in control culture medium, overnight. 16 hours later mediums were aspirated, cells were washed two times with pre-warmed PBS and the medium was replaced with either serum-free selenium-adequate or serum-free selenium-deficient medium and incubated for 3 days. At day 3, indicated concentrations (ranging from 25 μM to 200 μM) of H_2O_2 were added directly to the culture medium. Cells were cultured 4 more hours under these conditions and the required experiments are performed. Percentage of apoptotic cells and % of cells under oxidative stress were calculated.

3.4.2 Detection of Oxidative Stress in HCC Cell Lines

Huh-7, Hep3B-TR, HepG2 and HepG2-2.2.15 cells were seeded on sterile coverslips in 6-well plates. HCC cell lines were cultured at 37°C under 5% CO_2 in control culture medium overnight. Next day, the medium was replaced with either serum-free selenium-adequate or serum-free selenium-deficient DMEM or HAM's and the cells were cultured for 96 or 72 hours. The cells were washed with pre-warmed PBS for 3 times and incubated with DCFH-DA reaction mixture for 15 minutes at 37˚C. Cells were washed 3 times with pre-warmed PBS and analyzed under fluorescent inverted microscope. The non-fluorescent DCFH-DA can diffuse cell membranes into the cytoplasm where it is deacetylated and entrapped as DCFH (2′-7′-dichlorodihydrofluorescein) form. Creation of highly fluorescent product, DCF consequent to the oxidation of DCFH facilitates the detection of the cells exposed to oxidative stress under fluorescence microscope (Richter *et al.*, 1993). The cells that were cultured for 72 hours in selenium-adequate and selenium-deficient DMEM were treated for 4 hours with indicated concentrations of H_2O_2 (ranging from 25 μM to 200 μM) which was directly added to the culture medium before detection of oxidative stress. For Vitamin E

experiments, cells were treated with a total of 100 μM Vitamin E for 72 hours (1st day 50 μM, 2nd day and 3rd day 25 μM Vitamin E is added to culture medium) or 48 hours (1st day 50 μM, 2nd 50 μM) and then tested for oxidative stress by oxidant-sensitive fluorescent dye DCFH-DA on the 4th or the 3rd day of the culture under an inverted fluorescent microscope, respectively.

3.4.3 Detection and Counting of Death Cells

Huh7, Hep3B-TR, HepG2 and HepG2-2.2.15 cells were seeded on sterile coverslips in 6-well plates. HCC cell lines were cultured at 37°C under 5% CO_2 in control culture medium overnight. Next day, the medium was replaced with either serum-free selenium-adequate or serum-free selenium-deficient DMEM and the cells were checked under an inverted light microscope on daily basis up to 8 days and simultaneously, the nuclei of death cells and live cells were counted with Hoechst counter-staining. The culture medium was aspirated, the cells were washed with ice-cold PBS and incubated with ice cold absolute methanol for 10 minutes. Then methanol was discarded, cells were washed again with ice-cold PBS, and incubated with nuclear Hoechst 33258 fluorochromo dye for 5 minutes in dark. Then, Hoechst 33258 was aspirated and destaining was performed in ddH_2O water for 10 minutes. Next, coverslips were directly taken out from the well and excess water was removed by tissue paper. Finally, coverslips were mounted onto slides containing 20 μl 50% glycerol to be analyzed under fluorescence microscopy.

3.4.4 Apotosis Assays

3.4.4.1 TUNEL

TUNEL assay was performed using In Situ Cell Death Detection Kit according to manufacturer's recommendations. Cells were seeded on sterile coverslips in 6-well plates. HCC cell lines were cultured at 37°C under 5% CO_2 in control culture medium overnight. Next day, the medium was replaced with either serum-free selenium-adequate or serum-free selenium-deficient DMEM. At the 4th day of the culture the medium was aspirated, and cells were washed with ice-cold PBS.

Then, cells were fixed with 1 ml 4% paraformaldehyde for 30 minutes at room temperature, and kept 2 minutes in 1 ml permeabilization solution on ice. After that, cells were washed with PBS, placed on stretch film, and incubated with 50 µl of TUNEL reaction mixture at 37°C for 1 hour. Following this, cells were washed with PBS and ddH_2O and counter stained with Hoechst 33258, mounted and visualized under fluorescent microscope.

3.4.4.2 Annexin V

Annexin V assay was performed using Annexin V-PE reagent on live cells according to the manufacturer's recommendations. Cells were seeded on sterile coverslips in 6-well plates. HCC cell lines were cultured at 37°C under 5% CO_2 in control culture medium overnight. Next day, the medium was replaced with either serum-free selenium-adequate or serum-free selenium-deficient DMEM. At the 4^{th} day of the culture the medium was aspirated, and cells were washed with ice-cold PBS, and rinsed with 1 ml binding buffer. Next, the coverslips were placed on stretch film, and treated with 100 µl of 10% Annexin V-PE diluted in binding buffer for 20 minutes in dark at room temperature. Following steps were avoided from direct light exposure. The coverslips were placed back to the wells, washed with binding buffer, and fixed in 1 ml 70% cold ethanol for 30 minutes on ice. Counter staining was performed with Hoechst 33258, coverslips were mounted and visualized under fluorescent microscope.

3.4.4.3 NAPO Assay

For NAPO assay (Sayan *et al.*, 2001b) cells that were grown on sterile coverslips at 37°C under 5% CO_2 in control culture medium overnight. Next day, the medium was replaced with either serum-free selenium-adequate or serum-free selenium-deficient DMEM or HAM's. At the 4^{th} day of the culture the cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature, and then rinsed and permeabilized with permeabilization buffer on ice for 10 minutes. After washing with PBS-T, cells were blocked with 3% BSA in PBS-T for 1 hour at room temperature and then incubated with anti-NAPO monoclonal antibodies

(1:1) for an hour at room temperature. Subsequent to washing with PBS-T, the cells were incubated with FITC-conjugated anti-mouse secondary antibody for 1 hour at room temperature, washed 3 times with PBS-T. Counter staining was performed with Hoechst 33258 nuclear staining, Then, Hoechst 33258 was aspirated and destaining was performed in double-distilled water for 10 minutes. Coverslips were directly taken out from the well and excess water was removed by tissue paper. Finally, the coverslips were mounted up side down onto slides containing 20 µl 50% glycerol. Samples were visualized under fluorescence microscopy.

3.4.5 Immunofluorescence

Huh7, Hep3B-TR, HepG2 and HepG2-2.2.15 cells were seeded on sterile coverslips in 6-well plates. HCC cell lines were cultured at 37°C under 5% CO_2 in control culture medium overnight. Next day, the medium was replaced with either serum-free selenium-adequate or serum-free selenium-deficient DMEM or HAM's. 4 days later, the growth medium was aspirated, the cells were washed gently with ice-cold PBS and fixed with 4% paraformaldehyde for 30 minutes at room temperature, rinsed and permeabilized in ice-cold acetone for 10 minutes. After washing with PBS, the cells were blocked with 3% BSA in PBS-T for 1 hour at 37°C and then incubated with anti-cytochrome c monoclonal antibody overnight (1:100; Achenbach *et al.*, 2000). Next day, after washing with PBS-T, the cells were incubated with FITC-conjugated secondary antibody (1:100) for 1 hour at room temperature in dark, washed 3 times with PBS-T, incubated with Hoechst 33258 for 5 minutes to have nuclear counter-staining. Then, Hoechst 33258 was aspirated and destaining was performed in double-distilled water for 10 minutes. Coverslips were directly taken out from the well and excess water was removed by tissue paper. Finally, the coverslips were mounted up side down onto slides containing 20 µl 50% glycerol. Samples were analyzed by fluorescence microscopy.

3.4.6 Western Blot

Huh7, Hep3B-TR, HepG2 and HepG2-2.2.15 cells were seeded on 100 cm tissue culture plates and cultured at 37°C under 5% CO_2 in control culture medium overnight. Next day, the medium was replaced with either serum-free selenium-adequate or serum-free selenium-deficient medium.

3.4.6.1 Cell Lysis and Crude Total Proteins

At the 4th day of the culture in serum-free selenium-adequate or serum-free selenium-deficient DMEM, the medium of adherent monolayer Huh-7, Hep3B-TR, HepG2 and HepG2-2.2.15 cells was aspirated, cells were washed with ice-cold PBS, scraped in 1 ml PBS on ice, centrifuged at 13000 rpm for 10 minutes, resuspended in 4 times the volume of NP-40 (Igepal) lysis buffer, and incubated on ice for 30 minutes. The lysate was centrifuged at 13000 rpm for 10 minutes at 4˚C, the supernatant was quantified by Bradford assay, aliquoted, liquid nitrogen frozen and kept at -80°C.

3.4.6.2 Bradford Assay

In order to perform Bradford assay (Bradford, 1976), BSA was prepared at a concentration of 1 mg/ml, aliquoted, and stored at -20°C. Different concentrations of BSA, as shown below, were used to obtain a standard curve to find out the concentration of the crude protein extract obtained.

Tubes	b	1	2	3	4	5
ddH_2O (µl)	100	99	98	97	96	95
BSA (µl)	0	1	2	3	4	5
Bradford working solution (µl)	900	900	900	900	900	900

b: blank

In parallel, protein samples were prepared by mixing 900 µl bradford working solution with ddH_2O (90-98 µl) and sample (2-10 µl) in a tube. The volumes of ddH_2O and sample that are used depend on the concentration of the sample. The darker the color of the bradford reaction mixture means the more concentrated the sample. For concentrated samples, high volume of ddH_2O is used. In brief, after mixing protein and ddH_2O in 1.5 ml tubes, bradford working solution was added on the samples, vortexed and incubated for 5 minutes at room temperature. Immediately, absorbance of the samples was read at 595 nm using a UV-visible spectrophotometer (595 nm: visible) in disposable cuvettes. By using Microsoft Excel graphic program, a standard curve is obtained, BSA concentrations on x-axis and absorbance on y-axis, with a line formula of y=mx+c. The concentrations of the samples of interest are calculated according to the line formula.

3.4.6.3 SDS-PAGE

8 % resolving (lower) SDS-PAGE solution was prepared as in the table below.

Components	Volume (ml)
24% Acrylamide mix	1.65
Lower Gel Buffer	2.5
ddH_2O	0.85
10% APS	0.075
TEMED	0.003

As soon as TEMED was added, the gel solution was poured between the glasses of vertical gel electrophoresis system adjusted according to the manufacturer's recommendations (EC 160, E-C Apparatus Corp., Holbrook, NY, USA), leaving about 2 cm space for the comb and the stacking (upper) solution. After the gel solution was poured, immediately 2-propanol, which was equilibrated with equal volume of ddH_2O, was added on the solution between the glasses to have a straight and bubble-free resolving (lower) gel and it was allowed to polymerize

for 45 minutes. Following it, 2-propanol was washed off by ddH_2O and the stacking (upper) SDS-PAGE solution was prepared as in the table below.

Components	Volume (ml)
24% Acrylamide mix	0.375
Upper Gel Buffer	1.5
ddH_2O	1.125
10% APS	0.05
TEMED	0.003

The stacking gel solution was poured as soon as TEMED was added, and the comb was placed between the glasses and the gel was again allowed to polymerize for 30 minutes.

Meanwhile, samples were prepared to have equal concentration in equal volume, and were solubilized in 1X cracking buffer, denatured at 100°C for 5 minutes, spinned down and 10 μg of protein was loaded through the wells and 3 μl of pre-stained marker was loaded in one well as a molecular weight control. Samples were run at 80 V until they pass stacking gel and enter to resolving gel. Once they entered, voltage was increased to 120 V and the run lasted 2 hours and next semi-dry transfer of the proteins were performed.

3.4.6.4 Semi-Dry Transfer of the SDS-PAGE Separated Proteins

While the proteins were separated according to their molecular weights on SDS-PAGE, transfer membranes were prepared. 4 pieces of 3M Whattman paper and PVDF (for PARP) or nitrocellulose (for Akt) membranes were cut as the same size as the resolving gel. They were soaked into a Tris-glycine transfer buffer containing cup and left for 30 minutes with the exception that PVDF was soaked into methanol and then ddH_2O for 1 minute each before being put into the Tris-glysine transfer buffer. After the run of the protein gel was over, two of the Whattman papers were put on Transblot-Semi Dry eletroblotting apparatus,

avoiding the bubbles, then the membrane, the gel, and finally the last two Whattman papers were put on top of each other, again avoiding the bubbles. Transfer of the proteins from the gel to the PVDF western blotting membrane was performed at 12 V for 1 hour and at 8 V for 1 hour, and the transfer of the proteins from nitrocellulose was performed at 0.23 A for 40 minutes.

3.4.6.5 Detection of the PVDF or Nitrocellulose Immobilized Protein of Interest

The membranes were blocked for 1 hour at room temperature with slow shaking, and incubated with mouse anti-PARP monoclonal antibody (1:250) for 1 hour at room temperature. Unbound antibody was washed four times with TBS-T. The membrane was then incubated with HRP conjugated secondary antibody (1:1500) for 1 hour at room temperature, washed and detected by ECL detection kit according to the manufacturer's recommendations. The chemiluminescence emitted was captured on X-ray film in 10 minutes. Equal loading control was determined by probing the same membrane with mouse anti-CK18 monoclonal antibody.

3.4.7 Giemsa Staining

Cells plated in tissue culture under indicated conditions are washed with PBS for 4 times, and directly stained with Giemsa which is diluted 1:5 with ddH_2O for 5 minutes. Then, cells were washed 5 times with ddH_2O by slow shaking and analyzed under light microscopy.

3.4.8 Polymerase Chain Reaction

For the amplification of target sequences, HBsAg and HBxAg genes of HBV genome, a reaction mixture containing 1 u Taq polymerase, 1.5 mM $MgCl_2$, 5 mM dNTP, 20 pmol forward and reverse primers, 5 µl from 10X buffer, and 50 ng DNA template is prepared and completed to 50 µl with ddH_2O. 5 minutes hot start at 95˚C, a 30 cycle of 30 seconds denaturation at 95˚C, 30 seconds annealing at 58˚C, and 45 seconds extension at 72˚C is performed. The reaction ended with a

final extension and cooling of 5 minutes at 72˚C and 4˚C, respectively. The primer sequences used is given in table 3.4.8.1.

Table 3.4.8.1: Primer Sequences.

Target Gene	Primer Sequence
HBsAg	F 5'CCCAATACCACATCATCC3' R 5'GGATTGGGGACCCTGCGC3'
HBxAg	F 5'CTGGATCCTGCGCGGGACGTCCTT3' R 5'GTTCACGGTGGTCTCCAT3'

CHAPTER 4. RESULTS

The association of selenium-deficiency with hepatic disease conditions including hepatocellular carcinoma is well documented, however the underlying molecular mechanisms of this association are poorly characterized. In order to discover the phenomena lying under the malignant type of response of hepatocytes to selenium-deficient conditions, an *in vitro* experimental model with total loss of Gpx activity under selenium-deficient conditions using HCC-derived human cell lines, Huh-7 and Hep3B-TR have been established (İnce, 1999). Gpx activity has been recovered upon selenium supplementation. It has been demonstrated that Huh-7 cells responded to this deficiency with apoptosis, but Hep3B-TR cells did not, which was unexpected.

The available data from literature related to the link between selenium-deficiency, loss of Gpx activity and generation of oxidative stress led us to determine the intracellular oxidative stress in Huh-7 cells under selenium-deficient conditions and compare it with selenium-adequate conditions, which is a negative control.

4.1 Analysis of Oxidative Stress in Huh-7 Cell Line

Huh-7 cells were cultured in control medium overnight, and the next day the medium of the cells was replaced with selenium-adequate and selenium-deficient DMEM or HAM's medium. As demonstrated in Figure 4.1.1, on the 4^{th} day of the culture in DMEM, the expected accumulation of ROS due to selenium-deficiency induced loss of Gpx activity was detected at the cellular level in some live Huh-7

cells with the oxidant-sensitive non-fluorescent dye DCFH-DA that gives a fluorescence signal upon oxidation to DCF (Richter *et al.*, 1993). The remaining live cells were non-fluorescent, most probably indicating time-dependent differential response of the cells under the same conditions. On the other hand, Huh-7 cells were entirely non-fluorescent under selenium-adequate DMEM medium as expected since these cells maintain Gpx activity under selenium-adequate experimental conditions. This data was in complete correlation with the literature that relates loss of Gpx activity in the liver of animals that were subjected to *in vivo* selenium-deficiency induced oxidative stress (Fu *et al.*, 1999; Cheng *et al.*, 2003). The healthy looking non-apoptotic cells can be seen under selenium-adequate and selenium-deficient conditions in the light picture. Those live cells with fluorescent signal under selenium-deficiency will most probably die soon.

Figure 4.1.2 clearly illustrates the accumulation of ROS in Huh-7 cells that were cultivated in HAM's medium on the 3rd day of the culture under selenium-deficiency. As in the case of DMEM, under selenium-deficiency, some cells displayed a fluorescent signal, a direct indication of accumulated ROS in live cells, while other live cells did not. It appeared that ROS accumulation was faster in Huh-7 cells that were cultured under HAM's medium as compared to the cells that were cultured under DMEM. This was an expected outcome since Copper/Zinc that was present in HAM's medium had a key role for the function of Copper/Zinc SOD enzyme in converting $^{\cdot}O_2^{-}$ to H_2O_2 , which is a substrate for selenium-dependent Gpx enzyme (Matés, 2000). Dead cells were negative for the fluorescent signal under selenium-deficiency. On the other hand, Huh-7 cells were entirely non-fluorescent under selenium-adequate HAM's medium, as expected.

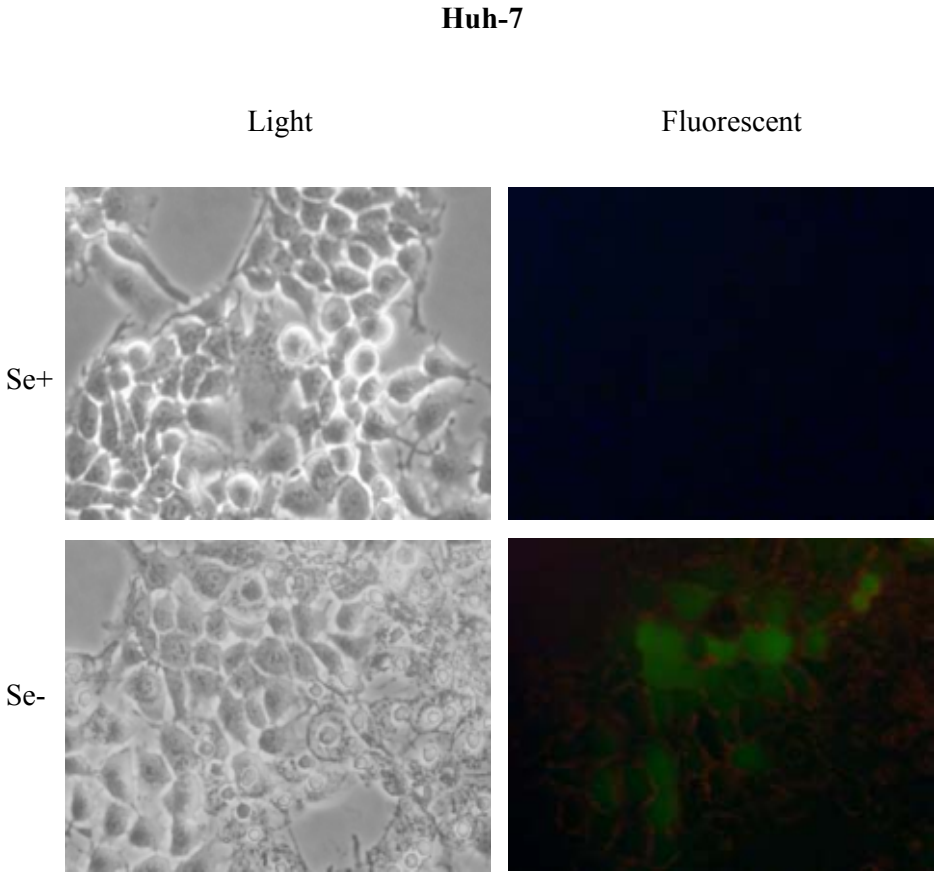

Figure 4.1.1: Analysis of oxidative stress in Huh-7 cell line by oxidant-sensitive fluorescent dye DCFH-DA in DMEM. Huh-7 cells were cultured in selenium-adequate (Se+) and selenium-deficient DMEM (Se-) medium for 96 hours. Presence of cells with fluorescence under selenium-deficient conditions indicates that those Huh-7 cells harbor oxidative stress (right panel). Light microscopy photographs (left panel) of the corresponding area of light-fluorescent photographs of Huh-7 cells under selenium-adequate and selenium-deficient conditions were also shown. Original magnification 200×.

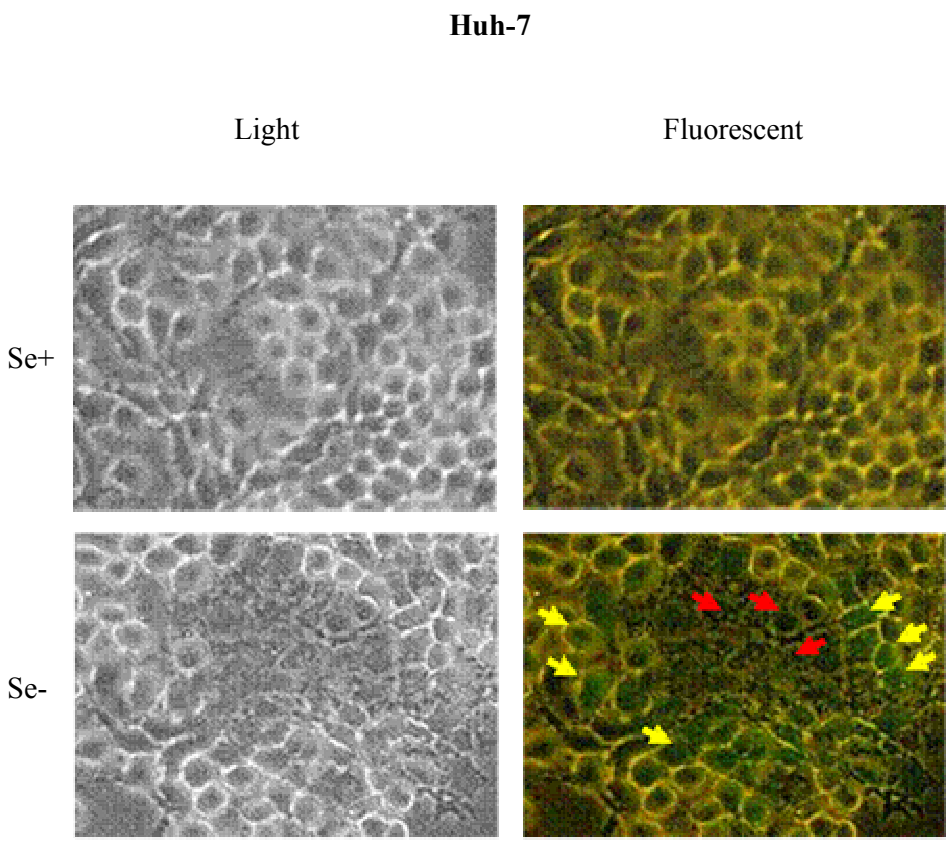

Figure 4.1.2: Analysis of oxidative stress in Huh-7 cell line by oxidant-sensitive fluorescent dye DCFH-DA in HAM's medium. Huh-7 cells were cultured in selenium-adequate (Se+) and selenium-deficient HAM's (Se-) medium for 72 hours. Presence of cells with fluorescence under selenium-deficient conditions indicates that those Huh-7 cells harbor oxidative stress (right panel). Light microscopy photographs (left panel) of the corresponding area of light-fluorescent photographs of Huh-7 cells under selenium-adequate and selenium-deficient conditions were also shown. Yellow arrows show the cells under oxidative stress and red arrows show apoptotic cells. Original magnification 200×.

4.2 Analysis of Survival of Huh-7 Cells under Selenium-Deficiency

With the observation of oxidative stress in Huh-7 cells that were cultured in DMEM or HAM's medium under selenium-deficient conditions, we wanted to see how their survival was impaired under the same conditions.

Firstly, in order to test this, Huh-7 cells were plated under control medium supplemented with 10% FBS overnight, and the next day the medium was replaced with selenium-adequate or selenium-deficient HAM's medium that contained 10 μM copper and 3mM zinc. The cells were analyzed in a daily manner under inverted light microscopy. The apparent morphologic changes were initially observed on the 2nd day of the culture under selenium-deficiency, while there was no change under selenium-adequate conditions. The light photography pictures in Figure 4.2.1 clearly shows the changes in the morphology of Huh-7 cells under selenium-deficient HAM's medium on the 3rd day of the culture, which reminded the apoptotic cells resulting from culture in selenium-deficient DMEM as shown in Figure 2.1.2.

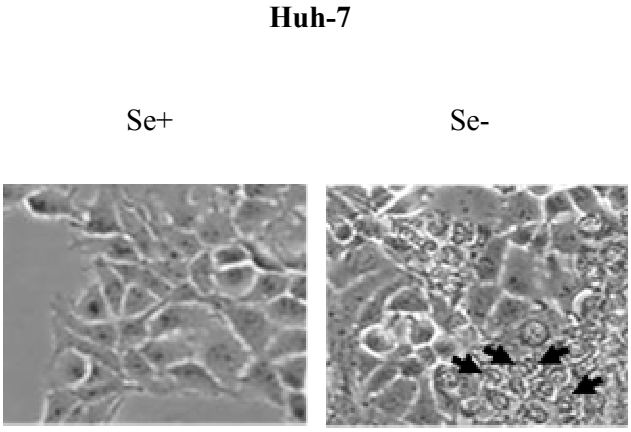

Figure 4.2.1: Death of Huh-7 cells under selenium-deficiency in HAM's medium. Huh-7 cells were examined under inverted light microscopy on a daily basis, and photographs of cells cultured under standard culture medium supplemented with selenium (Se+) and deprived of selenium (Se-) conditions were taken at day 3. Note the morphological changes of Huh-7 cells under selenium-deficient conditions as shown by black arrows. Original magnification 100×.

Also, we cultured Huh-7 cells in DMEM and extended their cultivation up to 8 days under selenium-adequate and selenium-deficient conditions. In DMEM, the cells were analyzed under light microscopy at different time intervals up to 8 days. In the first 2 days, there was no difference in the morphology of the cells under either condition. However, Huh-7 cells cultured under selenium-adequate and selenium-deficient conditions were growing slowly. This was expected since both selenium-adequate and selenium-deficient mediums were almost serum-free, which was leading the cells to slow proliferation. Starting from the 3rd day of the culture, some morphological changes were observed under selenium-deficient experimental conditions, while no such changes were present under selenium-adequate conditions. These evident alterations in the morphology were reminder of the changes that apoptotic cells displayed, such as nuclear chromatin condensation, cytoplasmic contraction, shrinking, and formation of membrane bound apoptotic bodies. On the 4th day, the number of cells with morphological changes under selenium-deficient conditions increased, while there was still no apparent morphological change under the other condition. Figure 2.1.2 clearly shows the changes in the morphology of Huh-7 cells under selenium-deficiency.

In order to have an idea of the percentage of the cells that die each day, condensed nuclei of the cells were counted daily after Hoechst 33258 nuclear staining (See Materials and Methods). Using Microsoft Excel, a graph that shows percentage cell death versus day was plotted as shown in Figure 4.2.2. Huh-7 cells that were cultured under selenium-adequate and selenium-deficient DMEM were counted up to 8 days. Under selenium-deficient conditions, the gradual increase in the number of the cells that died can be clearly seen from the graphic, whereas under selenium-adequate conditions, cells were protected and were fully alive even after 7 days. This difference in response of Huh-7 cells to two different growth media conditions suggests that availability of 0.1 μM selenium adds cells a property in coping with metabolically produced H_2O_2. But in the absence of this micronutrient, cells were disabled to cope with metabolically produced H_2O_2 and consequently died progressively in a time-dependent manner, death percentage reaching more than 70% at day 7.

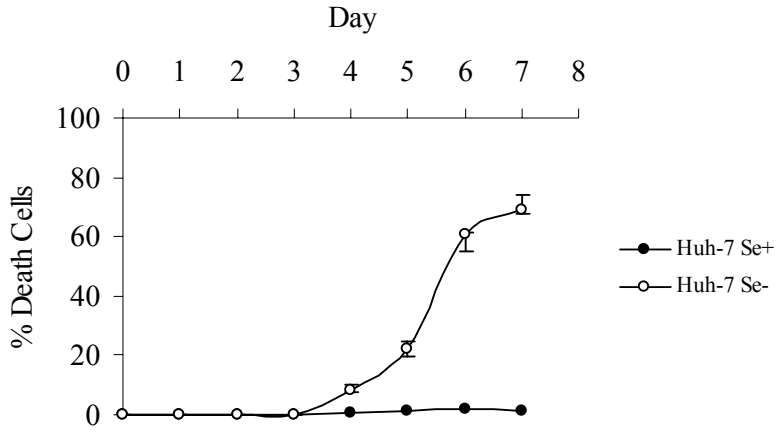

Figure 4.2.2: The percentage of death Huh-7 cells. Huh-7 cells were plated under selenium-adequate (Se+) and selenium-deficient (Se-) medium. The percentage of death Huh-7 cells was counted up to 8 days after Hoechst 33258 nuclear staining. Note the abundance of cell death in Huh-7 cells under Se- culture conditions. The results are from three separate experiments. Error bars indicate standard deviation.

4.3 Analysis of Apoptosis of Huh-7 Cells

Huh-7 cells that were cultured in DMEM under selenium-adequate and selenium-deficient conditions have been showed to die by apoptosis as explained in section 2.1. After that, we observed the accumulation of ROS in Huh-7 cells that were cultured in selenium-deficient DMEM and HAM's medium, observed the morphological changes, and we wanted to be sure that they die by apoptosis when cultured in HAM's medium like they did when they were cultured in DMEM. Moreover, apoptotic type of cell death in DMEM medium under selenium-deficiency was also confirmed.

This time instead of TUNEL or Annexin V assays, we used a recently described immunofluorescence technique, which detects the apoptotic cells that lose NAPO antigen. Figure 4.3.1 depicts that on the 4^{th} day of culture in selenium-deficient HAM's medium, Hoechst 33258-stained Huh-7 cells that have condensed nuclei

lost NAPO antigen as detected by a monoclonal antibody raised against NAPO antigen. On the contrary, non-apoptotic cells under both selenium-deficient and selenium-adequate condition retained NAPO expression. In addition to this, Huh-7 cells cultured in DMEM under selenium-deficient conditions for 4 days were also apoptotic as demonstrated by the loss of NAPO antigen in the dead cells as shown in Figure 4.3.2. On the contrary, Huh-7 cells that were cultivated in DMEM under selenium-adequate conditions had NAPO antigen. These data validated the apoptotic kind of cell death of Huh-7 cells under selenium-deficiency in both medium.

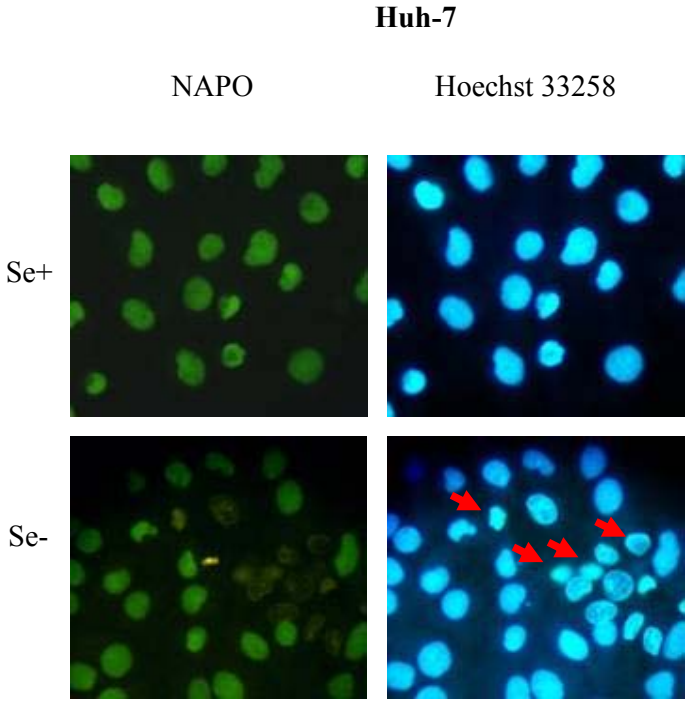

Figure 4.3.1: NAPO immunostaining of Huh-7 cells cultured in HAM's medium. Cells were cultured in selenium-adequate (Se+) and selenium-deficient (Se-) HAM's medium. Left panel shows the NAPO immunostaining and right panel shows the nuclear Hoechst 33258 staining of the corresponding area of NAPO staining. Note the loss of NAPO antigen in apoptotic Huh-7 cells. Corresponding nuclei of the cells that lose NAPO antigen are condensed as shown by nuclear Hoechst 33258 (red arrows). Huh-7 cells under selenium-adequate conditions have NAPO antigen (upper panel).

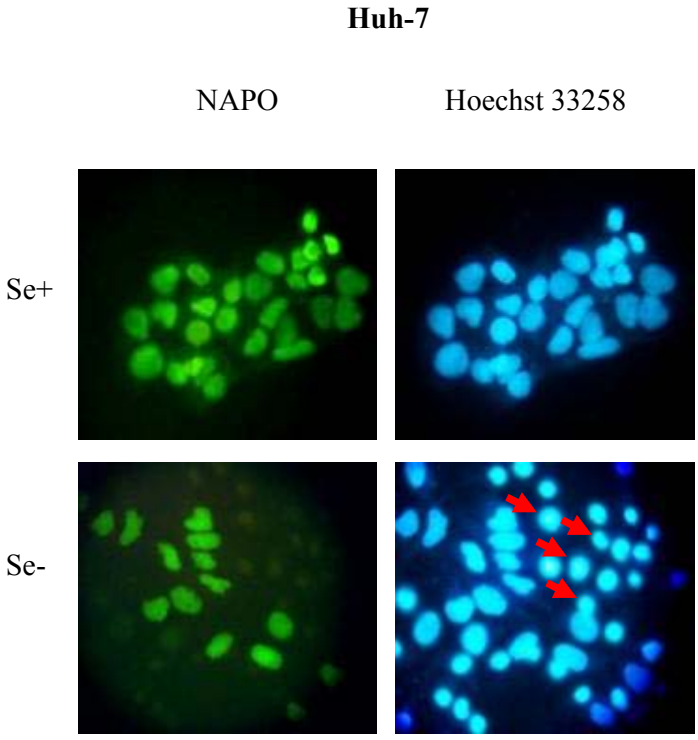

Figure 4.3.2: NAPO immunostaining of Huh-7 cells cultured in DMEM. Cells were cultured in selenium-adequate (Se+) and selenium-deficient (Se-) DMEM. Left panel shows the NAPO immunostaining and right panel shows the nuclear Hoechst 33258 staining of the corresponding area of NAPO staining. Note the loss of NAPO antigen in apoptotic Huh-7 cells. Corresponding nuclei of the cells that lose NAPO antigen are condensed as shown by nuclear Hoechst 33258 (red arrows). Huh-7 cells under selenium-adequate conditions have NAPO antigen (upper panel).

Until now, we have demonstrated that selenium-deficiency induces apoptotic cell death in Huh-7 cells with gradual increase in the number of the apoptotic cells in a daily manner and selenium-deficiency causes generation of oxidative stress in these cells. Apoptotic cell death is often accompanied with the release of cytochrome c from mitochondria to the cytoplasm (Liu *et al.*, 1996; Li *et al.*, 1997). Depending on these data that we have obtained, to understand the molecular mechanism under this apoptotic response, we wanted to analyze the

release of cytochtome c protein from the intermembrane space of mitochondria to cytoplasm. The release of cytochrome c from mitochondria to cytoplasm upon peroxidation of cardiolipin, which is strongly associated with cytochrome c, has been recently reported (Shidoji *et al.*, 1999).

Huh-7 cells were cultured in DMEM under selenium-adequate and selenium-deficient conditions for 4 days and analyzed by immunostaining of cytochrome c as shown in Figure 4.3.3. Under selenium-deficient condition, apoptotic Huh-7 cells had diffuse cytoplasmic stain, which was an indication of the release of cytochrome c in apoptotic cells, and the non-apoptotic cells under the same conditions had punctuated mitochondrial stain,. On the other hand, Huh-7 cells under selenium-adequate conditions had sole punctuated mitochondrial stain like non-apoptotic cells under selenium-deficiency.

The release of cytochrome c from mitochondria to cytoplasm has been described to initiate a mitochondrial apoptotic cascade that involves activation of pro-caspase 3 by cytochrome c (Liu *et al.*, 1996). In the cytosol, ctochrome c binds to the apoptotic protease-activating factor 1 (Apaf-1), which interacts with dATP and pro-caspase 9 to form the apoptosome complex (Li *et al.*, 1997). Subsequent activation of pro-caspase 9 initiates a caspase cascade involving downstream executioners, such as caspase 3 and finally the cleavage of target proteins such as PARP (Kaufmann *et al.*, 1993).

Consequently, Huh-7 cells were cultured in DMEM under selenium-adequate and selenium-deficient condition until more than 90% of Huh-7 cells were apoptotic under the latter condition. Caspase 3 activity in these cells was analyzed by performing western blot analysis to detect levels of PARP protein as shown in Figure 4.3.4. Western blot analysis of Huh-7 cells indicated that under selenium-adequate conditions Huh-7 cells had intact PARP protein, as expected from the previous data, that is, these cells were negative for apoptotic markers such as TUNEL, Annexin V, and NAPO. As we have shown in Figure 4.3.3 cytochrome c release was not observed in these cells.

On the other hand, total proteolysis of PARP protein was observed under selenium-deficient conditions, which revealed that selenium-deficiency induced cytochrome c release, most probably due to the oxidative stress induced disruption of mitochondrial membrane, and subsequently activation of caspase 3 in Huh-7 cells. Equal loading control with probing with CK18 illustrated that equal amount of proteins was loaded to the gel and the loss of PARP protein was specific since there was no change in the level of CK18 protein under the experimental conditions. Thus, the great difference seen in PARP probing in Huh-7 cells was due to the PARP cleavage by activated caspase 3 under selenium-deficient conditions.

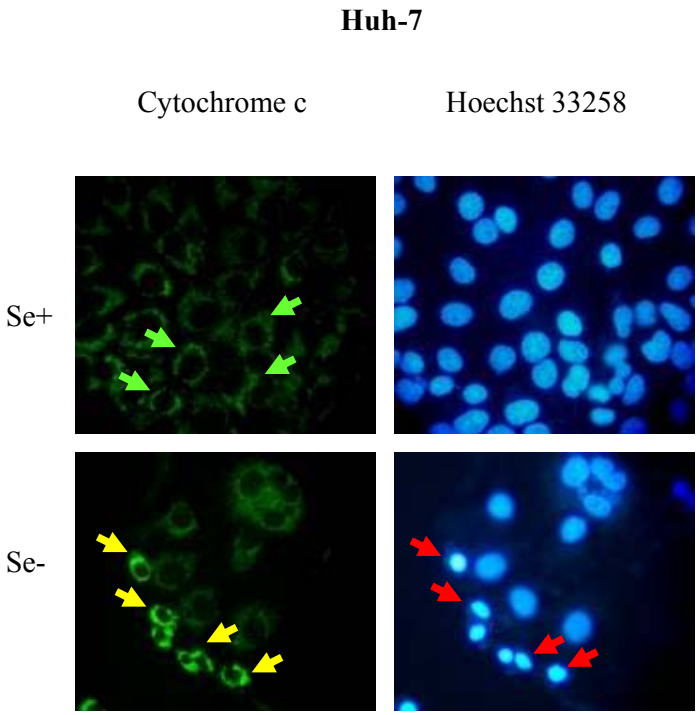

Figure 4.3.3: Cytochrome c immunostaining of Huh-7 cultured in DMEM. Cells were cultured in selenium-adequate (Se+) and selenium-deficient (Se-) DMEM for 4 days. Left panel shows the cytochrome c immunostaining and right panel shows the nuclear Hoechst 33258 staining of the corresponding area of cytochrome c staining. Note that under selenium-deficient conditions apoptotic Huh-7 cells display diffuse and more intense cytoplasmic stain (yellow arrows). Corresponding nuclei of cytochrome c releasing cells are condensed as shown by nuclear Hoechst 33258 staining (red arrows). Non-apoptotic cells under the same condition have punctuated mitochondrial cytochrome c stain. Under selenium-adequate conditions, Huh-7 cells have punctuated mitochondrial cytochrome c stain (green arrows).

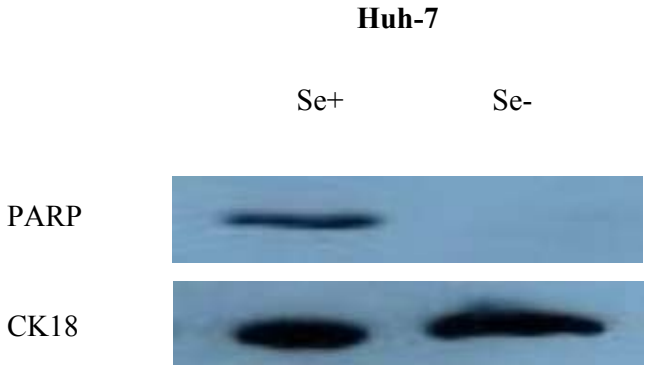

Figure 4.3.4: Western blot analysis of PARP protein in Huh-7 cells. Huh-7 cells were cultured in DMEM under selenium-adequate (Se+) and selenium-deficient (Se-) conditions. Upper panel shows PARP immunoblotting, and the lower panel shows CK18 immunoblotting, which is used as an equal loading control. Selenium-deficiency causes total proteolysis of PARP protein.

Taken together, the oxidative stress observed under selenium-deficient conditions elicited an apoptotic response in Huh-7 cells. However, to further survey a cause-effect correlation among selenium-deficiency, oxidative stress and subsequent apoptosis, we cultivated Huh-7 cells in either selenium-deficient or in selenium-adequate DMEM for 3 days and then treated them with increasing concentrations of H_2O_2. By this manner, we increased present internal oxidative stress under selenium-deficient conditions via introduction of exogenous oxidative stress. The cells were left in culture for an additional time of 4 hours, and the percentage of cells positive for ROS or apoptotic features were calculated. Huh-7 cells cultivated in a selenium-adequate medium were able to tolerate a treatment with at least 200 μM H_2O_2, with neither a sign of oxidative stress nor apoptosis; in contrast, selenium-deficient Huh-7 cells responded to H_2O_2 treatment by a dose-dependent increase in both oxidative stress and apoptotic cell death responses as illustrated in Figure 4.3.5. In the absence of exogenously added oxidative stress, about 20% of Huh-7 cells displayed oxidative stress and about 15% displayed apoptosis. 25 μM H_2O_2 was sufficient to cause an increase in oxidative stress with concomitant increase in apoptosis. At 200 μM H_2O_2 concentration, the cells under oxidative stress increased to about 40%. The percentage of apoptotic cells was

also about 40% at the same H_2O_2 concentration. This observation suggests that selenium-deficiency leads to sensitivity to oxidative stress, which in turn leads to apoptotic cell death.

All together, these observations indicate that *in vitro* selenium-deficiency in Huh-7 cells recapitulates hepatocellular injury observed in experimental animals, (loss of Gpx activity, oxidative stress, and cell death) exposed to selenium-deficiency (Cheng *et al.*, 2003). In addition, we present experimental proof that the cellular injury caused by selenium-deficiency occurs in the form of programmed cell death, at least *in vitro*. However, epidemiological studies clearly designate that selenium-deficiency is associated with an increased risk of HCC development, particularly in patients with chronic liver disease due to hepatitis B (Yu *et.al.*, 1997). At first sight, our observations would be against the expectation that selenium-deficiency will favor the expansion of HCC cells, rather than limiting their growth capacity by apoptotic cell death. As individual cell lines may vary in their phenotypic characteristics, and thus responses to certain experimental conditions, we aimed at carrying out similar studies in further HCC cell lines.

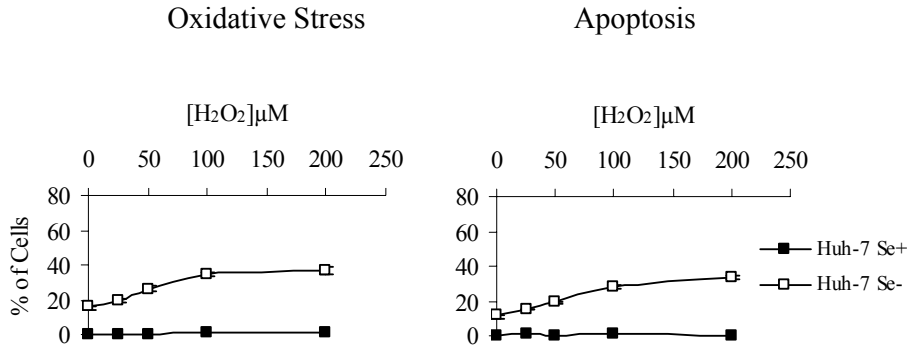

Figure 4.3.5: Correlation of oxidative stress with apoptosis in Huh-7 cells. Huh-7 cells were cultured in selenium-adequate (Huh-7 Se+) and selenium-deficient (Huh-7 Se-) DMEM for 72 hours and treated with indicated concentrations of H_2O_2. Four hours later, cells that are under oxidative stress are counted by inverted fluorescence microscope and apoptotic cells were counted by nuclear Hoechst 33258 staining. Note that Huh-7 Se- cells show time dependent increase in the percentage of cells under oxidative stress and apoptosis. The results are from three separate experiments. Error bars indicate standard deviation.

4.4 Analysis of Oxidative Stress in Hep3B-TR Cell Line

Introduction of selenium-deficiency and selenium-deficiency induced loss of Gpx activity in Hep3B-TR cells that were cultured in DMEM has been explained in section 2.1. The results were in correlation with a previous report demonstrating the time-dependent decrease in Gpx transcripts and activity in well-differentiated HCC-derived upon *in vitro* selenium-deficiency (Baker *et al.*, 1993).

We inspected the generation of oxidative stress in these cells under selenium-deficient conditions since they had no Gpx activity. Hep3B-TR cells were cultured for 4 days in DMEM under selenium-adequate and selenium-deficient conditions and subjected to DCFH-DA staining. Figure 4.4.1 points out that under selenium-adequate conditions Hep3B-TR cells displayed no fluorescence signal. This was expected as they had Gpx activity.

On the other hand, under selenium-deficiency, Hep3B-TR cells did not display any positive fluorescence signal, indicating that, unlike Huh-7 cells, they do not harbor oxidative stress. This was something unexpected since we estimated Hep3B-TR cells to have oxidative stress, like Huh-7 cells, as they both lost Gpx activity under our selenium-deficient experimental conditions. We would expect these cells to display oxidative stress since they have lost the activity of selenoprotein, Gpx, or to maintain Gpx activity in case of lack of oxidative stress. However, the outcome was like none of these two cases. They did not exhibit oxidative stress and did not have Gpx activity under selenium-deficiency. The light microscopic photograph of Hep3B-TR cells is also shown in the same figure.

Hep3B-TR cells were cultivated in HAM's medium under selenium-adequate and selenium-deficient conditions for 3 days, and were subjected to DCFH-DA staining. Figure 4.4.2 points out that under selenium-adequate conditions Hep3B-TR cells displayed no fluorescence signal. This was expected since their Gpx under this condition should be intact like in the case of culture in DMEM. On the other hand, under selenium-deficiency, Hep3B-TR cells did not display any

positive fluorescence signal even in HAM's medium that contains Copper/Zinc indicating that, unlike Huh-7 cells, they do not harbor oxidative stress.

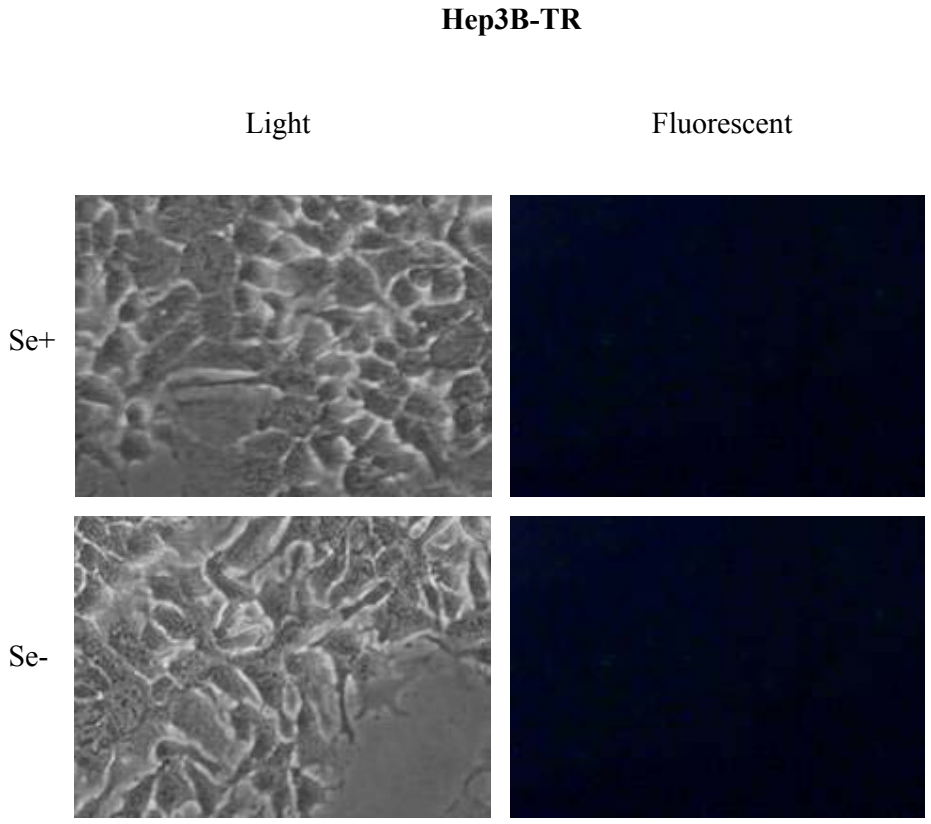

Figure 4.4.1: Analysis of oxidative stress in Hep3B-TR cell line by oxidant-sensitive fluorescent dye DCFH-DA in DMEM. Hep3B-TR cells were cultured in selenium-adequate and selenium-deficient DMEM for 96 hours. Absence of cells with fluorescence signal under selenium-deficient conditions indicates that Hep3B-TR cells do not harbor oxidative stress (right panel). Light microscopy photographs (left panel) of the corresponding area of fluorescent photographs of Hep3B-TR cells under selenium-adequate and selenium-deficient conditions were also shown. Original magnification 200×.

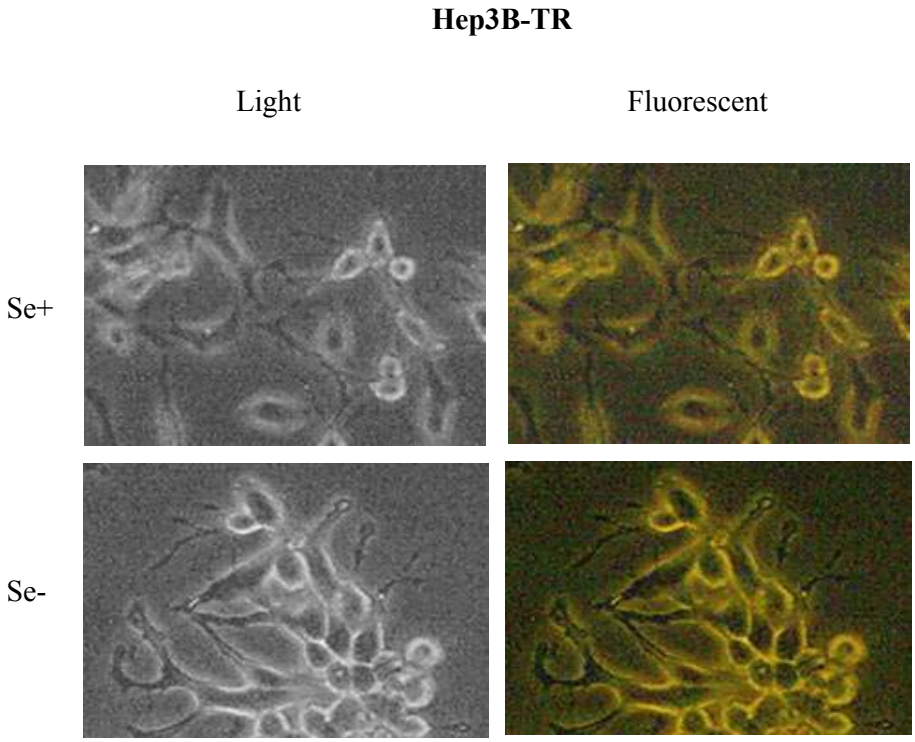

Figure 4.4.2: Analysis of oxidative stress in Hep3B-TR cell line by oxidant-sensitive fluorescent dye DCFH-DA in HAM's medium. Hep3B-TR cells were cultured in selenium-adequate (Se+) and selenium-deficient (Se-) HAM's medium for 72 hours. Absence of cells with fluorescence signal under selenium-deficient conditions indicates that Hep3B-TR cells do not harbor oxidative stress (right panel). Light microscopy photographs (left panel) of the corresponding area of fluorescent photographs of Hep3B-TR cells under selenium-adequate and selenium-deficient conditions were also shown. Original magnification 200×.

4.5 Analysis of Survival of Hep3B-TR Cells under Selenium-Deficiency

We illustrated that both Huh-7 and Hep3B-TR cells became selenium-deficient subsequent to 96 hour of selenium starvation in DMEM. Though, they differed sharply in their respective responses with respect to the exhibition of oxidative stress in both DMEM and HAM's medium.

To further analyze Hep3B-TR cells, we cultured them in control medium overnight, and next day replaced the medium with selenium-adequate or selenium-deficient HAM's medium that contains 10 μM copper and 3 mM zinc. The cells were analyzed in a daily manner under inverted light microscopy. As seen in Figure 4.5.1, in contrast to Huh-7 cells, Hep3B-TR cells did not display any morphological change under selenium-deficiency and seemed similar to the healthy growing Hep3B-TR cells under selenium-adequate conditions on the 3rd day.

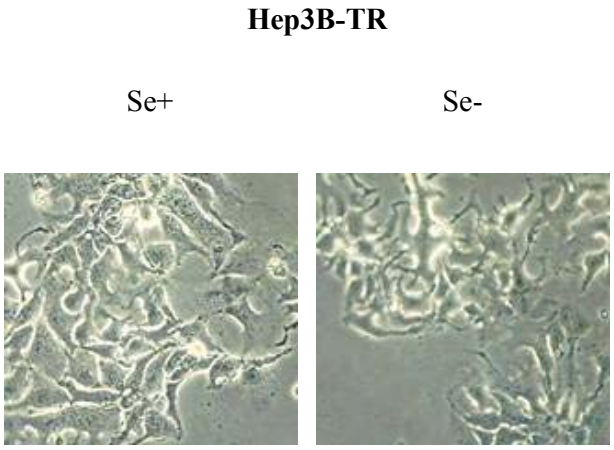

Figure 4.5.1: Survival of Hep3B-TR cells under selenium-deficiency in HAM's medium. Hep3B-TR cells are examined under inverted light microscopy on a daily basis, and photographs of Hep3B-TR cells under standard culture medium supplemented with selenium-adequate (Se+) and selenium-deficient (Se-) conditions were taken at day 3. Hep3B-TR cells did not show any morphological change under selenium-deficient conditions. Original magnification 100×.

In order to investigate whether Hep3B-TR cells exhibit any morphological changes and die at later time points, the nuclei of the cells were counted daily by Hoechst 33258 nuclear staining up to 8 days. Contrasting to Huh-7 cells, Hep3B-TR cells never showed any morphological changes and never had condensed nuclei that demonstrate dead cells. Using Microsoft Excel a graph that shows percents cell death versus day was plotted as shown in Figure 4.5.2. As seen in the graph, under selenium-deficient conditions, there were no dead Hep3B-TR cells even at the 7th day of the culture, the time at which more than 70% of Huh-7 cells were dead (compare Figure 4.2.2 and Figure 4.5.2).

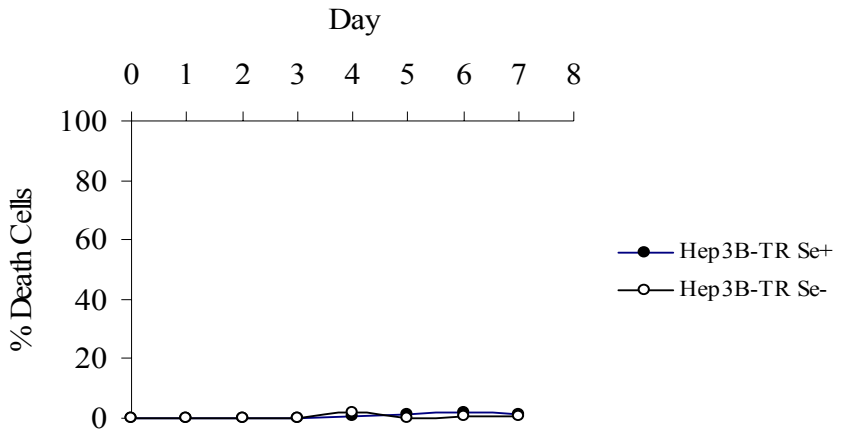

Figure 4.5.2: The percentage of death Hep3B-TR cells. The percentage of death Hep3B-TR cells cultured in DMEM under selenium-adequate (Se+) and selenium-deficient (Se-) was counted up to 8 days with Hoechst nuclear staining. Note the absence of dead Hep3B-TR cells under Se- culture conditions. The results were from three separate experiments. Error bars indicate standard deviation.

4.6 Analysis of Apoptosis of Hep3B-TR Cells

Briefly, under selenium-deficiency, Hep3B-TR cells with no Gpx activity neither harbored oxidative stress nor showed up any sign of morphological change and death. These facts suggested that Hep3B-TR cells could somehow tolerate selenium-deficiency induced oxidative stress and consequently escape apoptosis.

The lack of apoptosis in Hep3B-TR cells was demonstrated via TUNEL and Annexin V assays in section 2.1. After we observed the absence of accumulation of ROS in Hep3B-TR cells that were cultured in selenium-deficient HAM's medium and observed the proliferating healthy cells under inverted light microscopy, we decided to confirm the absence of apoptosis by NAPO assay. Figure 4.6.1 shows that, in contrast to Huh-7 cells (Figure 4.3.1), Hep3B-TR cells were negative for NAPO assay under selenium-deficiency in HAM's medium upon 4 day of culture. Also, as compared to Huh-7 cells, nuclear Hoechst staining of Hep3B-TR cells showed a different pattern with no condensed nuclei under selenium-deficiency. In parallel, we also confirmed that Hep3B-TR cells that were cultured in selenium-deficient DMEM were negative for apoptosis on the 4th day of the culture via NAPO assay as illustrated in Figure 4.6.2.

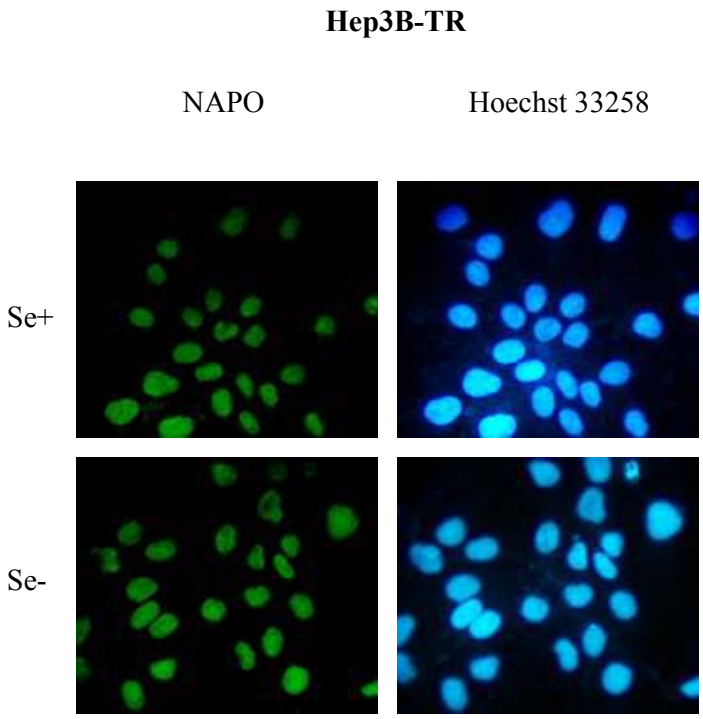

Figure 4.6.1: NAPO immunostaining of Hep3B-TR cells cultured in HAM's medium. Cells were cultured in selenium-adequate (Se+) and selenium-deficient (Se-) HAM's medium. Left panel shows the NAPO immunostaining and right panel shows the nuclear Hoechst 33258 staining of the corresponding area of NAPO staining. Note that Hep3B-TR cells are negative for NAPO assay under both conditions indicating that they are not apoptotic.

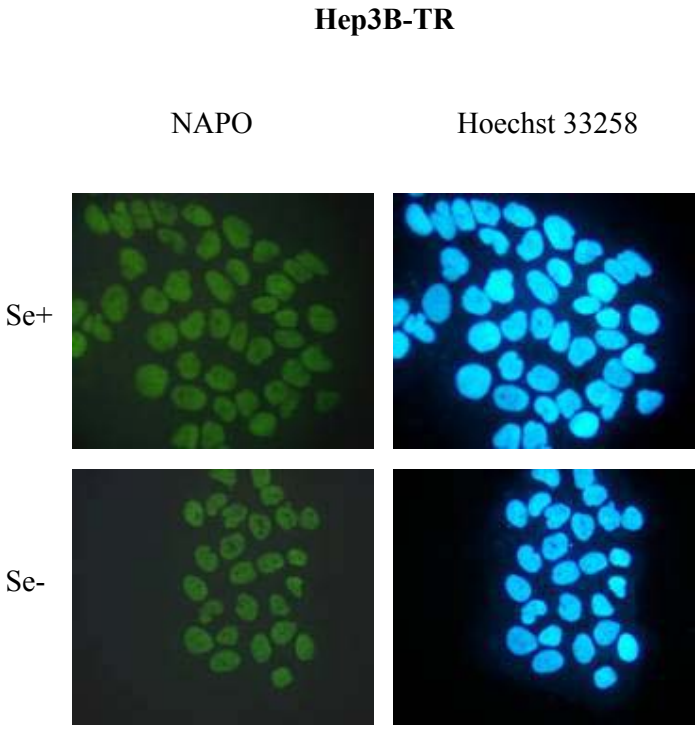

Figure 4.6.2: NAPO immunostaining of Hep3B-TR cells cultured in DMEM. Cells were cultured in selenium-adequate (Se+) and selenium-deficient (Se-) conditions. Left panel shows the NAPO immunostaining and right panel shows the nuclear Hoechst 33258 staining of the corresponding area of NAPO staining. Note that Hep3B-TR cells are negative for NAPO assay under both conditions indicating that they are not apoptotic.

Observing that Hep3B-TR cells lacked selenium-deficiency induced oxidative stress and that they were negative for apoptotic markers, we hypothesized that cytochrome c should be mitochondrial. As we suggested, Figure 4.6.3 shows the mitochondrial cytochrome c immunostaining of Hep3B-TR cells under selenium-adequate and selenium-deficient conditions at the 4^{th} day of the culture in DMEM, while at the same time point, cytochrome c was cytoplasmic in apoptotic Huh-7 cells (Figure 4.3.3). Thus, most probably the integrity of mitochondrial membrane of Hep3B-TR cells was not disrupted, attributable to the lack of oxidative stress and subsequent peroxidation of the membrane lipids under selenium-deficiency.

Hep3B-TR

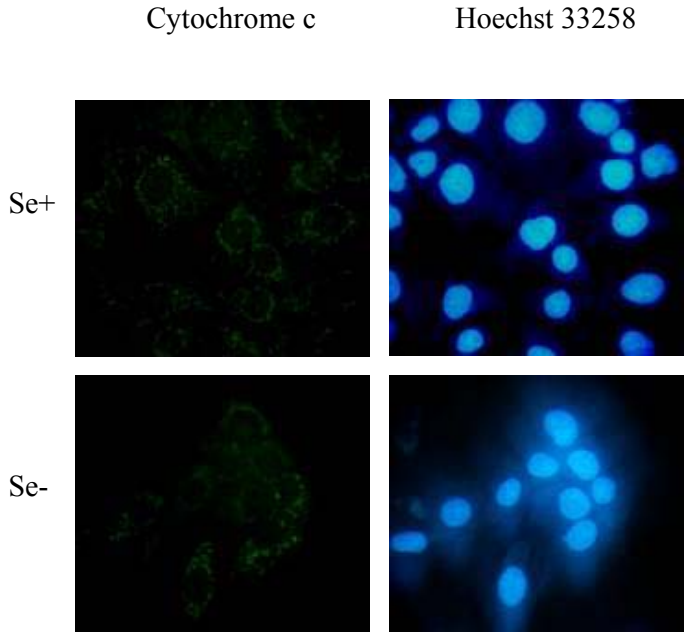

Figure 4.6.3: Cytochrome c immunostaining of Hep3B-TR cells cultured in DMEM. Cells were cultured in selenium-adequate (Se+) and selenium-deficient (Se-) DMEM for 4 days. Left panel shows the cytochrome c immunostaining and right panel shows the nuclear Hoechst 33258 staining of the corresponding area of cytochrome c staining. Note that cytochrome c has a punctuated mitochondrial immunostaining pattern under both conditions.

Accordingly, under selenium-deficient conditions, Hep3B-TR cells should not have caspase 3 activity, since its activation is related to the commitment of upstream apoptotic cascade. In our case, *in vitro* selenium-deficiency does not trigger apoptotic machinery in Hep3B-TR cells. Figure 4.6.4 depicts the immunoblot analysis of PARP protein, which is a caspase 3 target, under selenium-adequate and selenium-deficient conditions. The experiment was performed at the same time point when Huh-7 cells had more than 90% apoptosis. As expected, PARP protein is intact under both conditions. This implies that unlike Huh-7 cells, Hep3B-TR cells had inactive caspase 3 under selenium-deficiency. CK18 probing was performed as an equal loading control in this experiment.

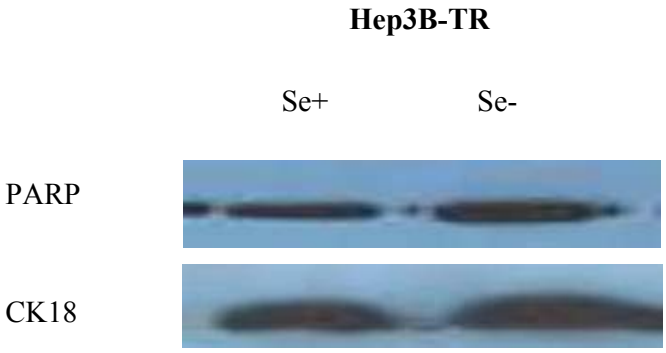

Figure 4.6.4: Western blot analysis of PARP protein in Hep3B-TR cells. Cells were cultured in selenium-adequate (Se+) and selenium-deficient (Se-) DMEM. Upper panel shows PARP immunoblotting, and the lower panel shows CK18 immunoblotting, which is used as an equal loading control. Note that PARP protein is intact under both conditions.

In summary, the lack of oxidative stress in selenium-deficient Hep3B-TR cells enabled these cells to be resistant to apoptosis. We thought that selenium-deficiency induced endogenous oxidative stress might not be sufficient to be detected and to trigger apoptosis. Consequently, we decided to introduce exogenous oxidative stress by directly adding increasing doses of H_2O_2 to the culture medium on the 3rd day of cultivation as it was performed for Huh-7 cells.

Under these conditions, Hep3B-TR cells with no Gpx activity should suffer from oxidative stress normally due to the fact that oxidative stress can not be eliminated by the selenoenzyme, Gpx. To analyze this situation, Hep3B-TR cells were plated in DMEM under selenium-adequate and selenium-deficient conditions. On the 3rd day of the culture, cells were treated with increasing concentrations of H_2O_2, the range being 25 to 200 μM. We have kept the maximum concentration of H_2O_2 at 200 μM to avoid necrotic cell death, since high doses of H_2O_2 has been reported to cause necrotic cell death (Palomba *et al.*, 1999). Cells were further cultured for 4 hours as in the case of Huh-7 cells, and analyzed for the presence of ROS and apoptotic cells by DCFH assay and Hoechst 33258 nuclear staining, respectively.

Under selenium-adequate conditions with increasing doses of H_2O_2, there was no oxidative stress and apoptosis in Hep3B-TR cells. To our surprise, there appeared no sign of oxidative stress and consequent apoptotic response of Hep3B-TR cells under selenium-deficient conditions even at the highest concentrations of externally introduced H_2O_2 as shown in the graphs in Figure 4.6.5. This revealed that Hep3B-TR cells were extremely tolerant to oxidative stress under selenium-deficient conditions, and even in case of exogenously introduced oxidative stress. This tolerance to oxidative stress under selenium-deficiency enabled Hep3B-TR cells to be resistant to subsequent apoptotic response.

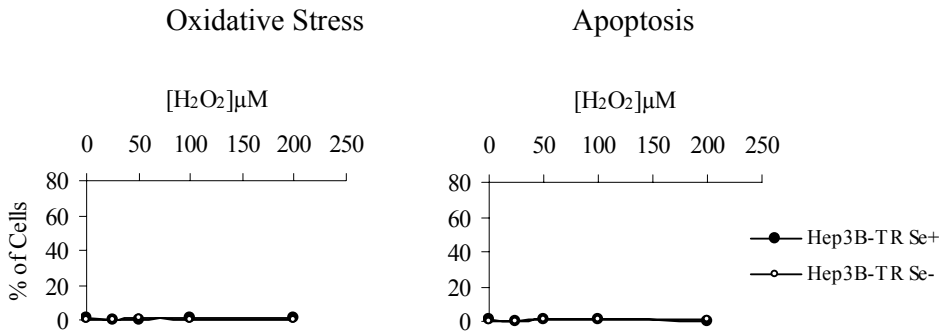

Figure 4.6.5: Treatment of Hep3B-TR cells with increasing doses of exogenous H_2O_2. Hep3B-TR cells were cultured under selenium-adequate (Hep3B-TR Se+) and selenium-deficient (Hep3B-TR Se-) conditions for 72 hours and treated with indicated concentrations of H_2O_2. Four hours later, cells that are under oxidative stress are counted by inverted fluorescence microscope and apoptotic cells were counted by Hoechst 33258 staining. Note that Hep3B-TR cells are free of oxidative stress and apoptosis even at the highest H_2O_2 concentration used under selenium-deficient conditions, implying the adaptation of these cells to eliminate ROS by selenium independent means. The results are from three separate experiments. Error bars indicate standard deviation.

The ability of Hep3B-TR cells to tolerate selenium-deficiency prompted us to compare the survival capacity of Huh-7 and Hep3B-TR cells. These two cell lines were cultivated in DMEM under selenium-adequate and selenium-deficient conditions for up to 11 days. Cells were checked under inverted light microscopy

and stained with Giemsa on the 10th day. Figure 4.6.6 illustrates clearly that selenium-deficient Hep3B-TR cells survive under these condition at least 10 days, since we can see Giemsa stained alive cells. On the other hand, there was no Huh-7 cell survived under these conditions for 10 days. Under selenium-adequate conditions, we can see both Huh-7 and Hep3B-TR cells stained with Giemsa, implying that they survived.

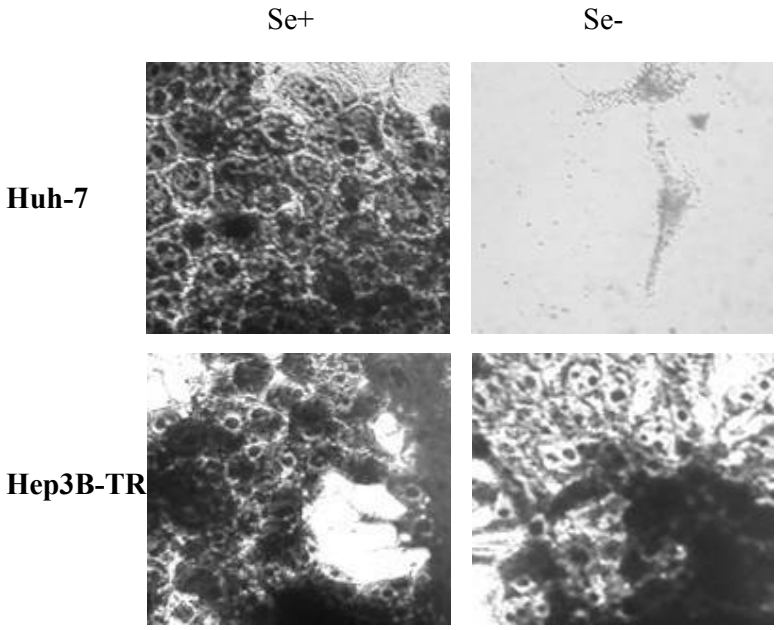

Figure 4.6.6: Comparison of the survival of Huh-7 and Hep3B-TR cells. Huh-7 (upper panel) and Hep3B-TR cells (lower panel) were cultured under selenium-adequate and selenium-deficient conditions for 10 days. Attached cells were visualized under inverted light microscopy by Giemsa staining. Note that the difference in survival is exhibited under selenium-deficient conditions. Under selenium-available conditions both cell lines survive, while under selenium-deficiency only Hep3B-TR cells survive. Original magnification 100×.

In brief, our experimental results concerning Hep3B-TR cells presented strong evidence that these cells are tolerant to selenium-deficiency induced oxidative

stress. Most probably, these malign HCC-derived cells have gained survival and subsequent proliferation capacity under long periods of selenium-deficiency. As evidenced by our H_2O_2 treatment experimental results, this acquired capacity to survive and proliferate is most likely to be due to their high tolerance to oxidative stress. Certainly, metabolic activity of these cells is preserved and continues to produce ROS under selenium-deficiency. So, the lack of oxidative stress even in the presence of excess H_2O_2 definitely indicates that they have adapted themselves to get rid of ROS by selenium-independent means. This ability provides them a selective survival advantage under selenium-deficient and oxidative stress conditions.

In addition we examined the activation of Akt, a downstream component of the PI3K pathway, which is important in regulating cell survival and apoptosis and influences cell survival upon oxidative injury (Ostrakhovitch *et al.*, 2002). Akt protein levels were viewed by western blot analysis under selenium-deficient or selenium-supplemented conditions with Huh-7 and Hep3B-TR cells. We observed no difference in neither unphosphorylated nor active phosphorylated Akt protein levels in these cell lines. In addition to our observations with DCFH assay, our data with Akt protein status indirectly indicates that under selenium-deficient conditions resistant Hep3B-TR cells do not display oxidative stress.

4.7 Rescue of Selenium-Deficient Huh-7 Cells from Oxidative Stress and Cell Death with Vitamin E

Accumulating data related to Vitamin E suggests that, Vitamin E is a potent protective scavenger against free radical damage. In the presence of metal ions, H_2O_2 is converted to $OH^{\cdot}$. Vitamin E which is incorporated into cellular membranes prevents the peroxidation of membrane lipids by reducing $OH^{\cdot}$ generated in the presence of metal ions and H_2O_2 (Claycombe and Meydani, 2001; Packer *et al.*, 2001).

We have demonstrated the accumulation of ROS, which in turn leads to apoptosis, in Huh-7 under selenium-deficient conditions and found out that Hep3B-TR cells

were resistant to the accumulation of ROS. Subsequently, we thought that, somehow, ROS induced apoptosis sensitive cell line, Huh-7, can become tolerant to selenium-deficiency induced ROS and subsequent apoptosis like Hep3B-TR cells.

Since Vitamin E has been demonstrated to be protective against generation of $OH^{\cdot}$, we wanted to test whether it protects Huh-7 cells from selenium-deficiency induced oxidative stress and apoptosis in Huh-7 cells via its scavenger antioxidant function. In order to illustrate this, Huh-7 cells were cultured in selenium-deficient DMEM for 4 days and the presence of oxidative stress was determined by DCFH assay. As shown in Figure 4.7.1 under selenium-deficient conditions, in the presence of Vitamin E Huh-7 cells did not show any accumulation of ROS as evidenced by the absence of fluorescent signal in the cells. In parallel, we did not observe any cell with apoptotic morphology.

However, under selenium-deficient conditions, in the absence of Vitamin E, ROS accumulation is clearly revealed by the presence of fluorescent cells in Figure 4.7.1. Moreover, cells with apoptotic morphology were also detected in the absence of Vitamin E.

These results show that Vitamin E has a protective antioxidant effect on selenium-deficiency induced ROS and consequent apoptosis. By this way we were able to revert apoptosis sensitive Huh-7 cells to apoptosis resistant cells under selenium-deficiency in the presence of Vitamin E.

Figure 4.7.2 shows that Vitamin E has indifferent effect on Hep3B-TR cells under the same conditions since we did not observe any oxidative stress in these cells under selenium-deficiency.

The same experiment was confirmed by using another source of Vitamin E on the 3^{rd} day of the culture as shown Figure 4.7.3 and Figure 4.7.4 for Huh-7 and Hep3B-TR, respectively.

Huh-7

Se-/Vit E - Se-/Vit E +

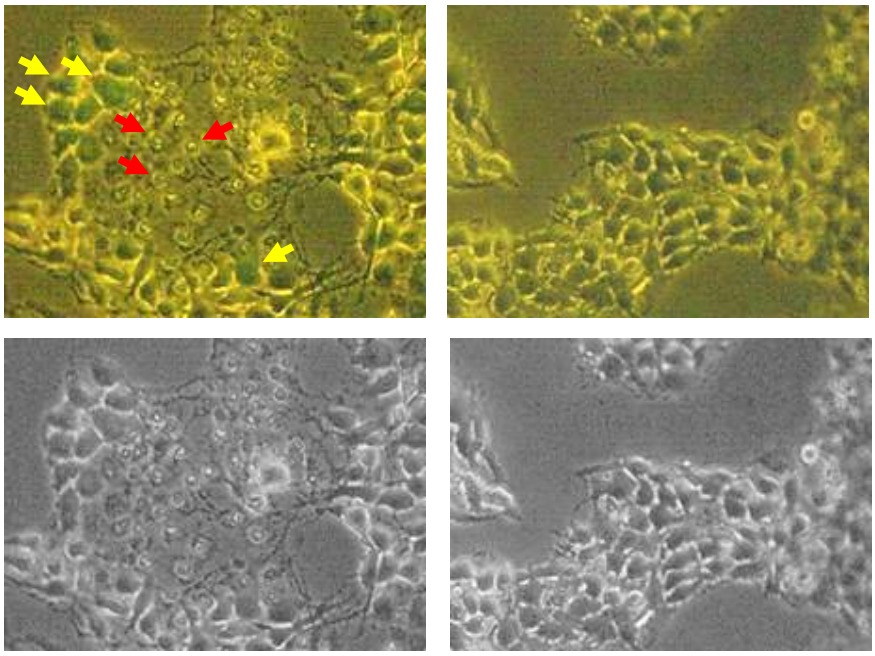

Figure 4.7.1: Resistance of Huh-7 cells to selenium-deficiency induced oxidative stress with Vitamin E. Huh-7 cells were cultured in selenium-deficient DMEM that lacks Vitamin E (Se-/Vit E-) or contains , Grandpherol® Vitamin E (Se-/Vit E+) and tested for oxidative stress by oxidant-sensitive fluorescent dye DCFH-DA under an inverted fluorescent microscope on the 4^{th} day of the culture. Presence of cells with fluorescence in the absence of Vitamin E indicates that they harbor oxidative stress (upper left panel, yellow arrows). Absence of cells with fluorescence in the presence of Vitamin E indicates that they do not harbor oxidative stress (upper right panel). The light photographs of fluorescent photographs are shown in the lower panel. Note the presence of apoptotic cells in the absence of Vitamin E (left panel, red arrows), and the absence of apoptotic cells in the presence of Vitamin E (right panel). Original magnification 200×.

Hep3B-TR

Se-/Vit E – Se-/Vit E +

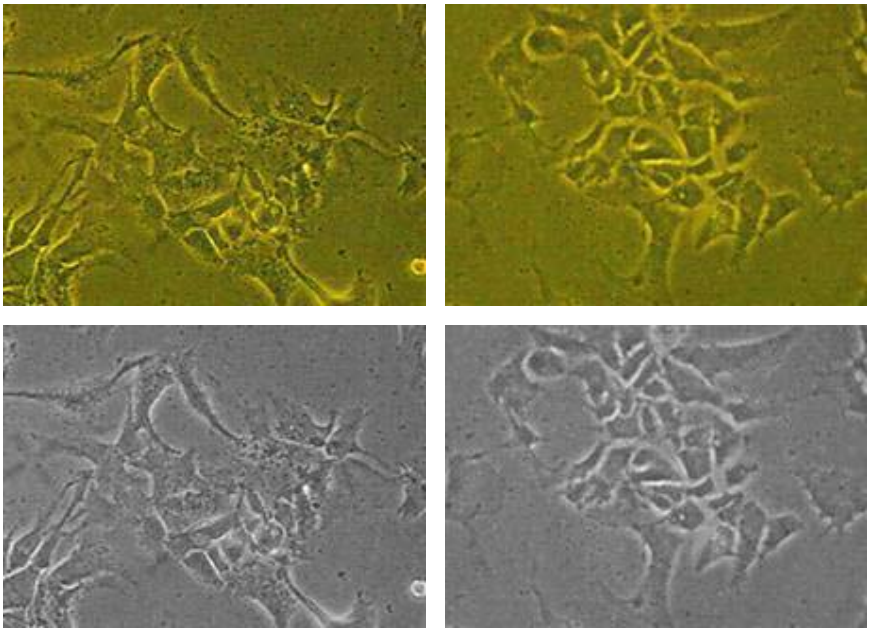

Figure 4.7.2: Indifferent effect of Vitamin E on Hep3B-TR cells under selenium-deficiency. Hep3B-TR cells were cultured under selenium-deficient DMEM that lacks Vitamin E (Se-/Vit E-) or contains Grandpherol® Vitamin E (Se-/Vit E+) and tested for oxidative stress by oxidant-sensitive fluorescent dye DCFH-DA under an inverted fluorescent microscope on the 4th day of the culture. There are no cells with fluorescence signal in the absence of Vitamin E (upper left panel) or in the presence of Vitamin E (upper right panel). Light photographs of the cells under both conditions are shown in the lower panel. Original magnification 200×.

Huh-7

Se-/Vit E – Se-/Vit E +

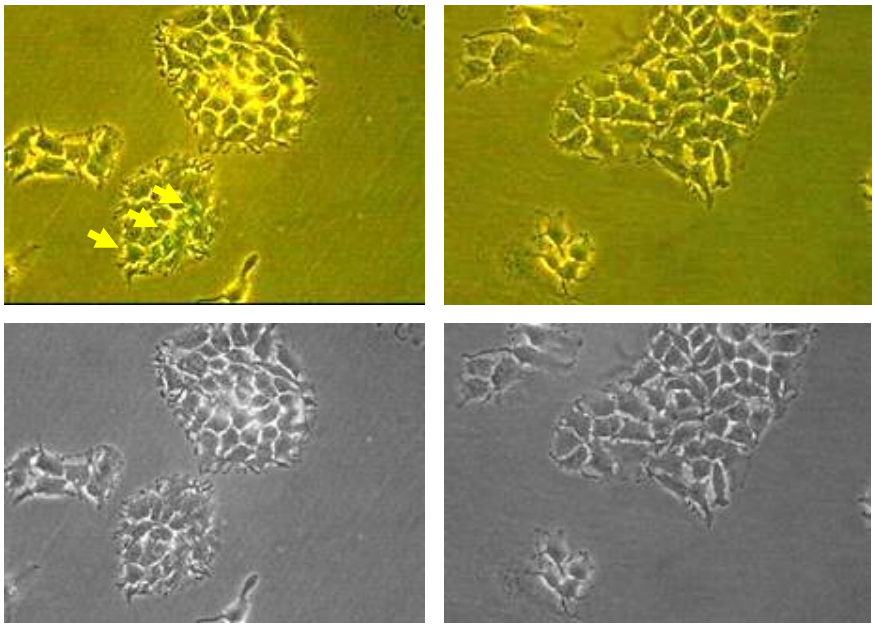

Figure 4.7.3: Confirmation of resistance of Huh-7 cells to selenium-deficiency induced oxidative stress with another source of Vitamin E. Huh-7 cells were cultured in selenium-deficient DMEM that lacks Vitamin E (Se-/Vit E-) or contains BASF Vitamin E (Se-/Vit E+) and tested for oxidative stress by oxidant-sensitive fluorescent dye DCFH-DA under an inverted fluorescent microscope on the 3th day of the culture. Presence of fluorescent Huh-7 cells in the absence of Vitamin E (upper panel, yellow arrows) indicates that they harbor oxidative stress. Absence of fluorescence in the presence of Vitamin E (upper right panel) indicates that Huh7 cells have acquired resistance to oxidative stress. Rare apoptotic cells were observe in the absence of Vitamin E since it is the 3rd day of the culture. Inverted light microscopy photographs (lower panel) of the corresponding area of fluorescent photographs of Huh7 cells are also shown. Original magnification ×200.

Hep3B-TR

Se-/Vit E – Se-/Vit E +

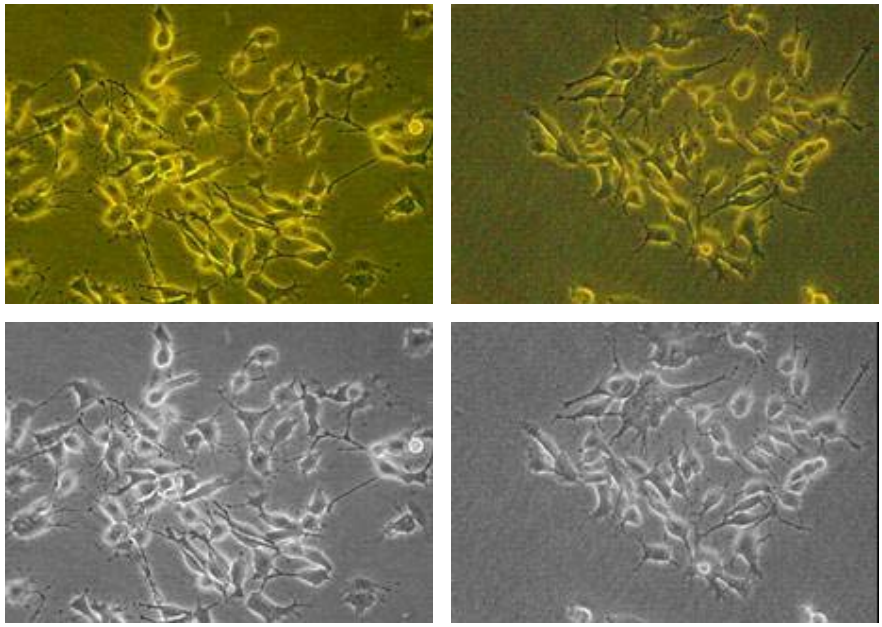

Figure 4.7.4: Indifferent effect of another source of Vitamin E on Hep3B-TR cells under selenium-deficiency. Hep3B-TR cells were cultured in selenium-deficient DMEM that lacks Vitamin E (Se-/Vit E-) or contains BASF Vitamin E (Se-/Vit E+) and tested for oxidative stress by oxidant-sensitive fluorescent dye DCFH-DA under an inverted fluorescent microscope on the 3rd day of the culture. There are no fluorescent Hep3B-TR cells in the absence of Vitamin E (upper left panel) or in the presence of Vitamin E (upper right panel) presence of fluorescent Huh-7 cells in the absence of Vitamin E (upper left panel) Inverted light microscopy photographs (lower panel) of the corresponding area of fluorescent photographs of Hep3B-TR cells are also shown. Original magnification 200×.

4.8 Most HCC Cell Lines are Tolerant to Selenium-Deficiency

The differential response of the Huh-7 and Hep3B-TR cell lines to selenium-deficiency encouraged us to extend our research to other HCC cell lines and examine them under selenium-adequate and selenium-deficient conditions. It would be interesting to find out at what extend the other cell lines exhibit the same tolerance to oxidative stress for the purpose of driving a relation to perhaps the etiology of HCC and the existing tolerant condition.

For this purpose, additional ten HCC-derived cell lines , namely, Hep40, Hep3B, SNU 423, SNU 387, SNU 182, FOCUS, SNU 475, SNU 449, PLC/PRF/5, and Mahlavu and one hepatoblastoma-derived cell line, HepG2 were cultured in standard DMEM supplemented with 10% FBS overnight. Next day, their medium was replaced with selenium-adequate and selenium-deficient medium. 9 of the 11 cell lines, namely, Hep3B, SNU 423, SNU 387, SNU 182, FOCUS, SNU 475, SNU 449, PLC/PRF/5, and Mahlavu, were able to grow under both conditions and survive and resist selenium-deficiency induced cell death. They were analyzed up to 11 days under inverted light microscopy in a daily manner and their photographs were taken at the 4^{th} day of cultivation as shown in Figure 4.8.1. On the other hand, there was a progressive and complete loss of the two cell lines, HepG2 and Hep40, like Huh-7 cells under selenium-deficiency upon culture for 4 days under selenium-deficiency as depicted in the same figure.

In summary, Table 4.8.1 shows that out of 13 cell lines examined, 10 were resistant to selenium-deficiency induced apoptosis. In contrast, 3 among 13 cell lines, namely, Huh-7, HepG2, and Hep40, were sensitive to selenium-deficient conditions, responding the selenium-deficiency induced oxidative stress by entering apoptosis. In other words, 77% of cell lines exhibited tolerance to oxidative stress thus gaining survival capacity, while the remaining 23% were not tolerant to oxidative stress under selenium-deficient conditions.

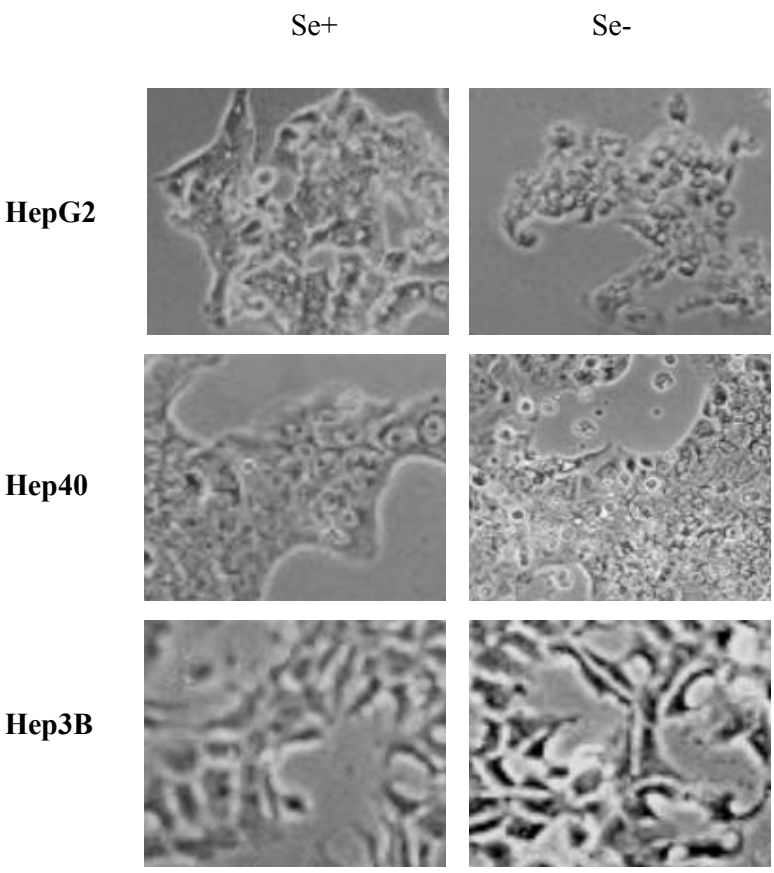

Figure 4.8.1: Survival analysis of HCC cell lines. Analysis of the survival of ten HCC-derived and one hepatoblastoma-derived cell lines cultured in DMEM under selenium-adequate (Se+) and selenium-deficient (Se) conditions. The cells were analyzed daily basis under inverted light microscope, and the photographs were taken at day 4. HepG2 (upper panel), Hep40 (middle panel), and Hep3B (lower panel) cell lines are shown. Under selenium-deficiency, the apoptotic HepG2 and Hep40 cells can be observed. Original magnification 100×.

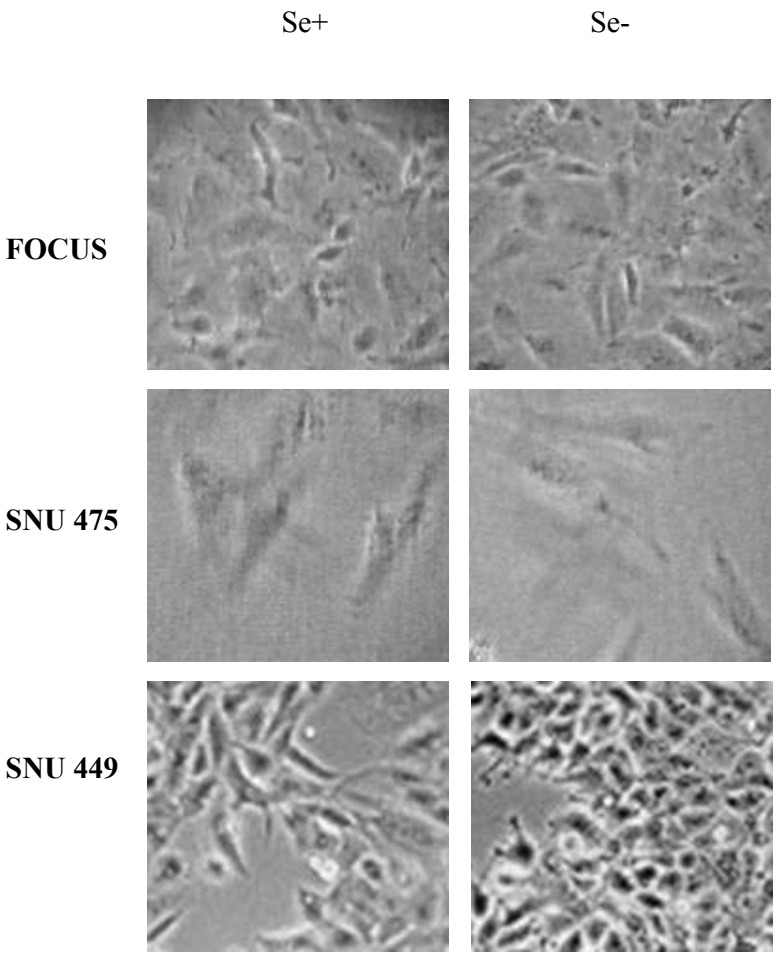

Figure 4.8.1 cont': Survival analysis of HCC cell lines. Analysis of the survival of ten HCC-derived and one hepatoblastoma-derived cell lines cultured in under DMEM under selenium-adequate (Se+) and selenium-deficient (Se-) conditions. The cells were analyzed daily basis under inverted light microscope, and the photographs were taken at day 4. FOCUS (upper panel), SNU 475 (middle panel), and SNU 449 (lower panel) cell lines are shown. Orignal magnification 100×.

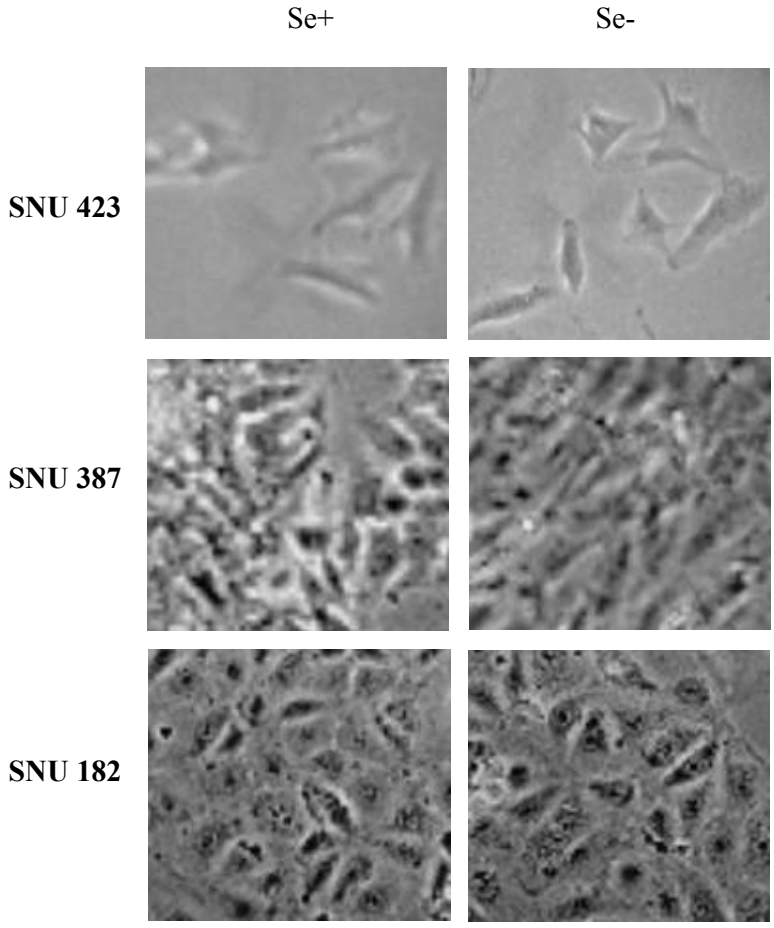

Figure 4.8.1 cont': Survival analysis of HCC cell lines. Analysis of the survival of ten HCC-derived and one hepatoblastoma-derived cell lines cultured in DMEM under selenium-adequate (Se+) and selenium-deficient (Se-) conditions. The cells were analyzed daily basis under inverted light microscope, and the photographs were taken at day 4. SNU 423 (upper panel), SNU 387 (middle panel), and SNU 182 (lower panel) cell lines are shown. Original magnification 100×.

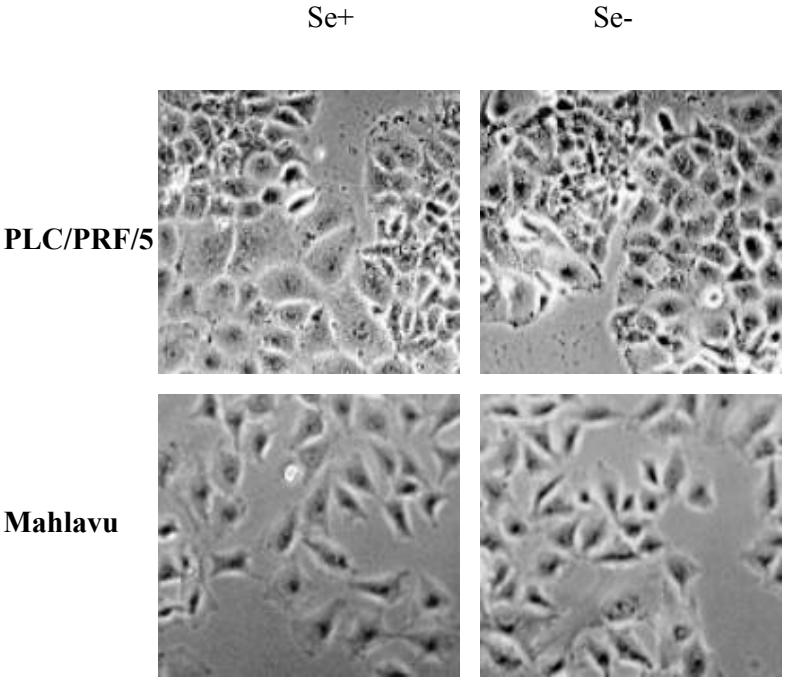

Figure 4.8.1 cont': Survival analysis of HCC cell lines. Analysis of the survival of ten HCC-derived and one hepatoblastoma-derived cell lines cultured in DMEM under selenium-adequate (Se+) and selenium-deficient (Se-) conditions. The cells were analyzed daily basis under inverted light microscope, and the photographs were taken at day 4. PLC/PRF/5 (upper panel), and Mahlavu (lower panel) cell lines are shown. Original magnification 100×.

The tolerant response of 10 out of 13 cell lines to selenium-deficiency induced oxidative stress led us to search the etiology behind their malignant phenotype. Since HBV infection is the major etiology in hepatocarcinogenesis, we compared the HBV sequence integration status of the cell lines that we analyzed.

90% of the resistant cell lines had past exposure to HBV. Table 4.8.1 summarizes the interesting association between the presence of HBV genome and tolerance to oxidative stress revealed under selenium-deficient conditions. It is noteworthy that the remaining resistant HCC-derived cell line, Mahlavu, exhibits an aflatoxin-related p53 mutation, again showing a history with a past exposure to this toxin. The synergistic effect of selenium-deficiency and HBV infection on hepatocellular carcinoma has been documented (Yu *et al.*, 1997). The remarkable association of tolerance to selenium-deficiency related oxidative stress with a background exposure to HBV suggested that these two could be linked. Either selenium-deficiency could increase the possibility of HBV infection in people, or HBV infection could facilitate the tumor cells to acquire tolerance to selenium-deficiency.

Table 4.8.1. Tolerance of Hepatocellular Carcinoma Cells to *in vitro* Selenium-Deficiency.

Cell Line	Tolerance	HBV DNA int.[a]	Other suspect etiology
Huh7	No	No	-
HepG2	No	No	-
Hep40	No	Yes	-
Hep3B	Yes	Yes	-
Hep3B-TR	Yes	Yes	-
FOCUS	Yes	Yes	-
SNU 475	Yes	Yes	-
SNU 449	Yes	Yes	-
SNU 423	Yes	Yes	-
SNU 387	Yes	Yes	-
SNU 182	Yes	Yes	-
PLC/PRF/5	Yes	Yes	Aflatoxins[b]
Mahlavu	Yes	No	Aflatoxins[b]

[a]Presence of Hepatitis B Virus DNA was tested by PCR amplification, as described previously (Ünsal *et al.*, 1994). These cell lines has been previously reported (Park *et al.*, 1995; Simon *et al.*, 1995).

[b]Cell line displaying p53-codon 249 mutation that has been associated to aflatoxin exposure (Bressac *et al.*, 1991; Hsu *et al.*, 1991).

4.9 HBV Confers Resistance to Selenium-Deficiency in HepG2 Cells

Next, we wanted to explore the interesting association between the presence of HBV genome and gaining resistance to selenium-deficiency induced oxidative stress more precisely. In addition to studying two different cell lines, one with HBV genome integration and the other with no HBV genome integration in detail, it would be more convincing to exploit the same issue on isogenic cell lines that have the same genetic background.

Therefore, we decided to use parental HepG2 cells and HBV-transfected 2.2.15 clone of HepG2 cells, designated as HepG2-2.2.15. HepG2-2.2.15 cell line has been produced by stably transfecting HepG2 cells with four tandem copies of the HBV genome. Infectious HBV virions have been reported to be produced from integrated viral sequences in this clone (Sells *et al.*, 1987). Before performing the experiments related to oxidative stress under selenium-deficiency, HBV-transfected HepG2-2.2.15 was analyzed for the presence of HBsAg and HBxAg genes of HBV genome and compared with its parental cell line, HepG2, by PCR analysis as shown in Figure 4.9.1. As clear from the figure, HepG2-2.2.15 cell line is positive for these sequences while HepG2 cell line is not.

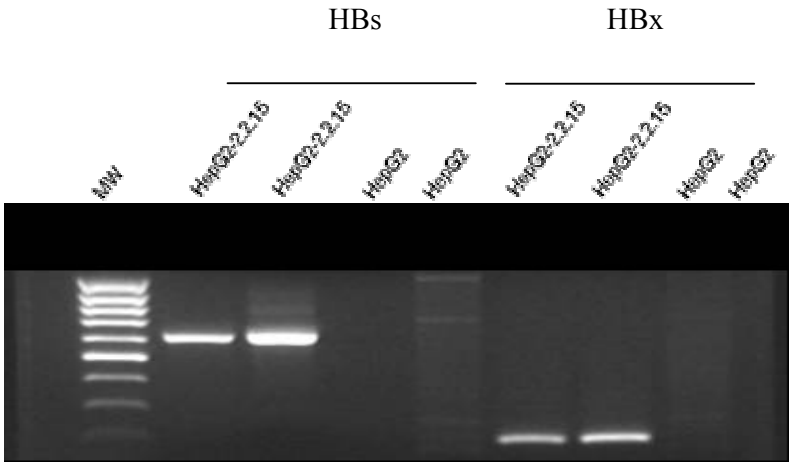

Figure 4.9.1: PCR analysis of isogenic cell lines. HepG2 and HepG2-2.2.15 cell lines are tested for the presence of and HBx genes of HBV genome. MW: 100 base pair molecular weight marker.

Next, we cultivated HepG2 and HepG2-2.2.15 cells in selenium-adequate and selenium-deficient DMEM for 10 days to compare their long-survival capacity. As depicted by Giemsa staining in Figure 4.9.2, both cell lines displayed similar growth capacity under selenium-adequate conditions as expected. Since in this experimental condition they were cultured under serum-free conditions, they do not detach due to over growth, and they are healthy looking. However, under selenium-deficient conditions the responses of the two isogenic cell lines were completely different. There were no live parental HepG2 cells, while HBV-positive HepG2-2.2.15 cells were still alive even at the 10^{th} day of the culture under selenium-deficient conditions.

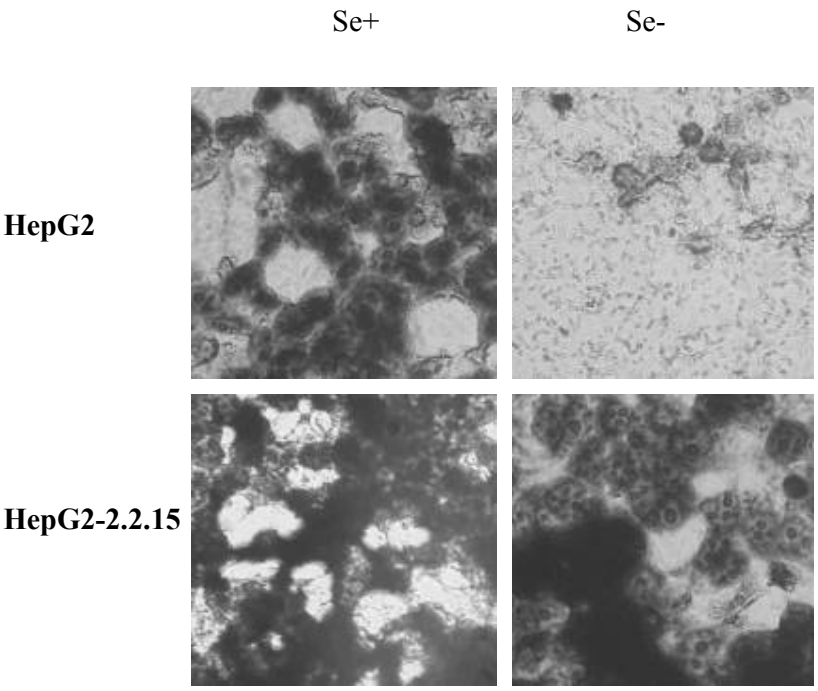

Figure 4.9.2: Comparison of the long-term survival of HepG2 and HepG2-2.2.15 cells. HepG2 (upper panel) and HepG2-2.2.15 cells (lower panel) were cultured in selenium-adequate and selenium-deficient DMEM for 10 days. Attached cells were visualized under inverted light microscopy by Giemsa staining. Note that the difference in survival is exhibited under selenium-deficient conditions. Under selenium-available conditions both cell lines survive, while under selenium-deficiency only HepG2-2.2.15 cells survive. Original magnification 100×.

We illustrated in Figure 4.8.1 that at the 4th day of the culture under selenium-deficiency, HepG2 cells exhibited different morphology when compared to the same cells cultured under selenium-supplemented conditions. Like we did for HepG2 cells previously, we cultivated HepG2-2.2.15 cells in DMEM supplemented with 10% FBS overnight and replaced the medium with selenium-adequate and selenium-deficient medium next day. As shown in Figure 4.9.3, unlike HepG2 cells, under selenium-deficiency, HepG2-2.2.15 cells were morphologically healthy and looked similar to the cells cultivated under selenium-adequate medium.

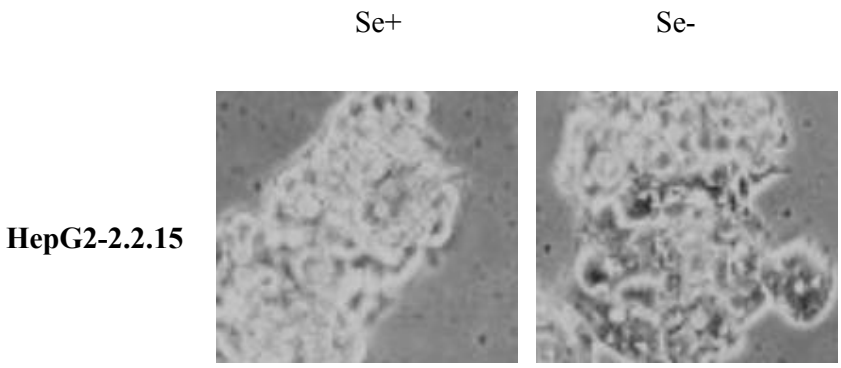

Figure 4.9.3: Survival of HepG2-2.2.15 cells under selenium-deficiency. HepG2-2.2.15 cells were examined under inverted light microscopy on a daily basis, and photographs of HepG2-2.2.15 cells cultivated in DMEM under selenium-adequate (Se+), and selenium-deficient (Se-) conditions were taken at day 4. HepG2-2.2.15 cells do not show any morphological change under selenium-deficient conditions. Original magnification 100×.

In parallel, we cultured HepG2 and HepG2-2.2.15 cells in selenium-adequate and selenium-deficient DMEM for 7 days and we counted the cells that die in a daily manner under inverted microscope by staining the cells with Hoechst 33258. Then, we calculated the percentage of the cells that died under selenium-adequate and selenium-deficient conditions, and finally plotted graphics showing the percentage of cell death versus day. Figure 4.9.4 illustrates that under selenium-supplemented conditions both cell lines survived. On the contrary, under selenium-deficient conditions there was a progressive loss of HepG2 cells reaching more than 70% at day 7, while HepG2-2.2.15 cells had no loss.

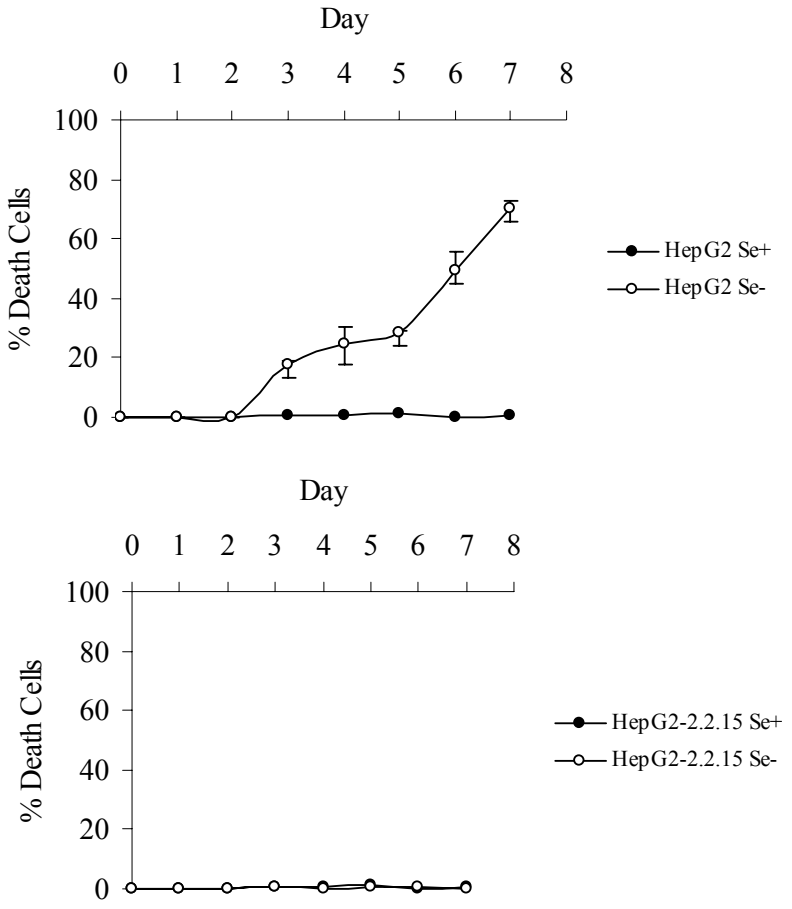

Figure 4.9.4: Analysis of percentage cell death of isogenic cell lines. The percentage of death HepG2 and HepG2-2.2.15 cells that were cultured in DMEM under selenium-adequate (Se+) and selenium-deficient (Se-) conditions was counted up to 8 days with Hoechst nuclear staining. Note the absence of death HepG2-2.2.15 cells under selenium-deficient culture conditions. The results are from three separate experiments. Error bars indicate standard deviation.

4.10 Oxidative Stress and Apoptosis in Isogenic Cell Lines

We cultivated HepG2 and HepG2-2.2.15 cells in selenium-adequate and selenium-deficient DMEM for 4 days. On the 4^{th} day of the culture under selenium-adequate and selenium-deficient DMEM, DCFH assay for HepG2 and HepG2-2.2.15 cells was performed to demonstrate the expected acquired tolerant behavior of HBV-positive HepG2-2.2.15 cells under selenium-deficient conditions.

As illustrated in Figure 4.10.1, fluorescence HepG2 cells were observed upon culture in DMEM under selenium-deficiency, implying the intracellular ROS in those cells. On the contrary, none of the HepG2-2.2.15 cells displayed fluorescence signal under selenium-deficiency. Light microscopic photographs of the fluorescence photographs are also shown in the same figure.

Under selenium-supplemented conditions, both cell lines were free of oxidative stress as expected most probably due to their active Gpx. This data suggested that HepG2-2.2.15 cells gained tolerance to oxidative stress generated under selenium-deficiency.

Similarly, Figure 4.10.2 illustrates the fluorescence of some HepG2 cells upon 3 days of culture in Copper/Zinc containing HAM's medium under selenium-deficient conditions, implying the accumulation of oxidative stress in most probably pro-apoptotic cells. Fluorescent cells were not present under selenium-adequate conditions. In addition to this, HepG2-2.2.15 cells were free of fluorescent cells and cell death under selenium-deficiency. These results imply that in the presence of H_2O_2 generated by Copper/Zinc SOD enzymes, HepG2-2.2.15 cells were still resisting the intracellular accumulation of ROS under selenium-deficiency.

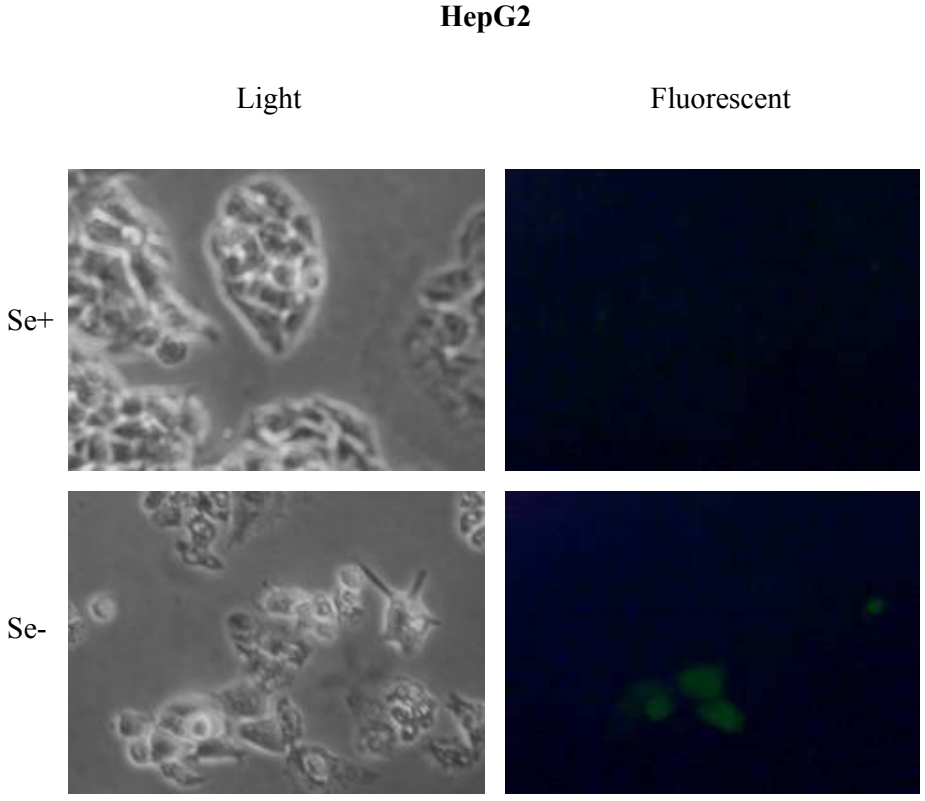

Figure 4.10.1: Analysis of oxidative stress in isogenic cell lines by oxidant-sensitive fluorescent dye DCFH-DA in DMEM. HepG2 cells were cultured under selenium-adequate (Se+) and selenium-deficient (Se-) DMEM for 96 hours. Presence of cells with fluorescence under selenium-deficient conditions indicates that those HepG2 cells harbor oxidative stress (right panel). Light microscopy photographs (left panel) of the corresponding area of fluorescent photographs of HepG2 cells under selenium-adequate and selenium-deficient conditions were also shown. Original magnification 200×.

HepG2-2.2.15

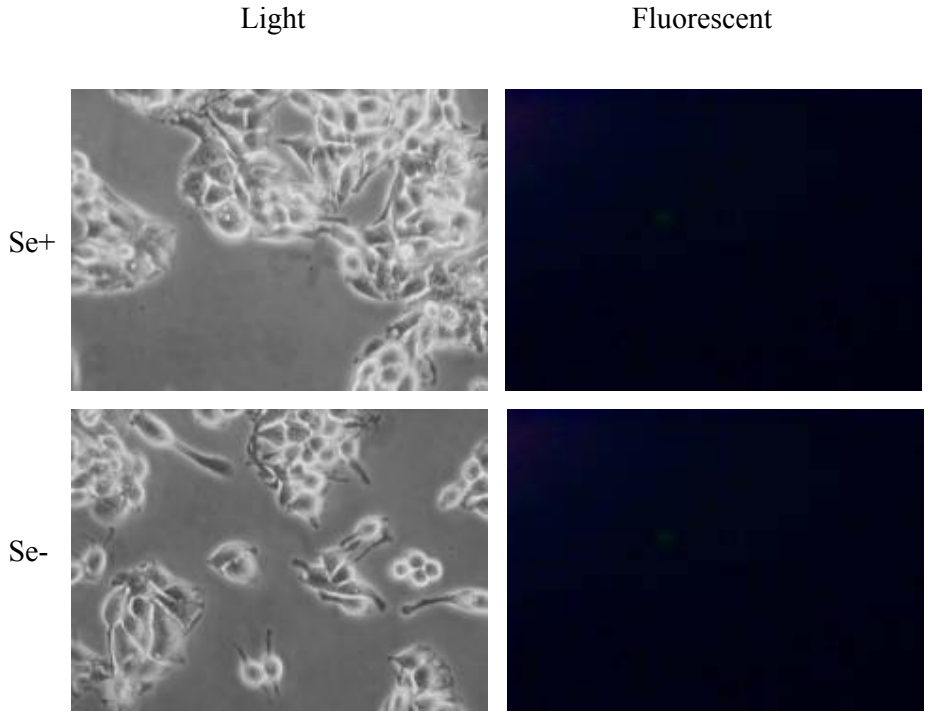

Figure 4.10.1 cont': Analysis of oxidative stress in isogenic cell lines by oxidant-sensitive fluorescent dye DCFH-DA in DMEM. HepG2-2.2.15 cells were cultured under selenium-adequate (Se+) and selenium-deficient (Se-) DMEM for 96 hours. Absence of cells with fluorescence signal under selenium-deficient conditions indicates that HepG2-2.2.15 cells do not harbor oxidative stress (right panel). Light microscopy photographs (left panel) of the corresponding area of fluorescent photographs of HepG2-2.2.15 cells under selenium-adequate and selenium-deficient conditions were also shown. Original magnification 200×.

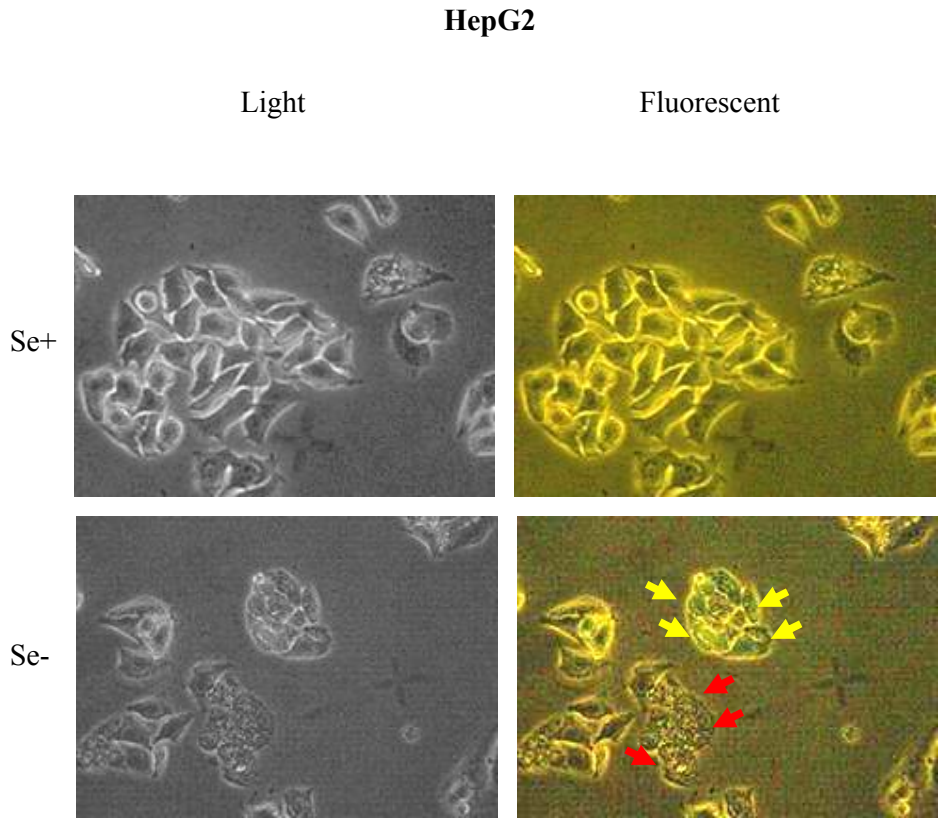

Figure 4.10.2: Analysis of oxidative stress in isogenic cell lines by oxidant-sensitive fluorescent dye DCFH-DA in HAM's medium. HepG2 cells were cultured under selenium-adequate (Se+) and selenium-deficient (Se-) conditions for 72 hours. Presence of fluorescent cells under selenium-deficient conditions indicates that those HepG2 cells harbor oxidative stress (right panel, yellow arrows). Light microscopy photographs (left panel) of the corresponding area of fluorescent photographs of HepG2 cells under selenium-adequate and selenium-deficient conditions were also shown. Red arrows show the apoptotic HepG2 cells. Original magnification 200×.

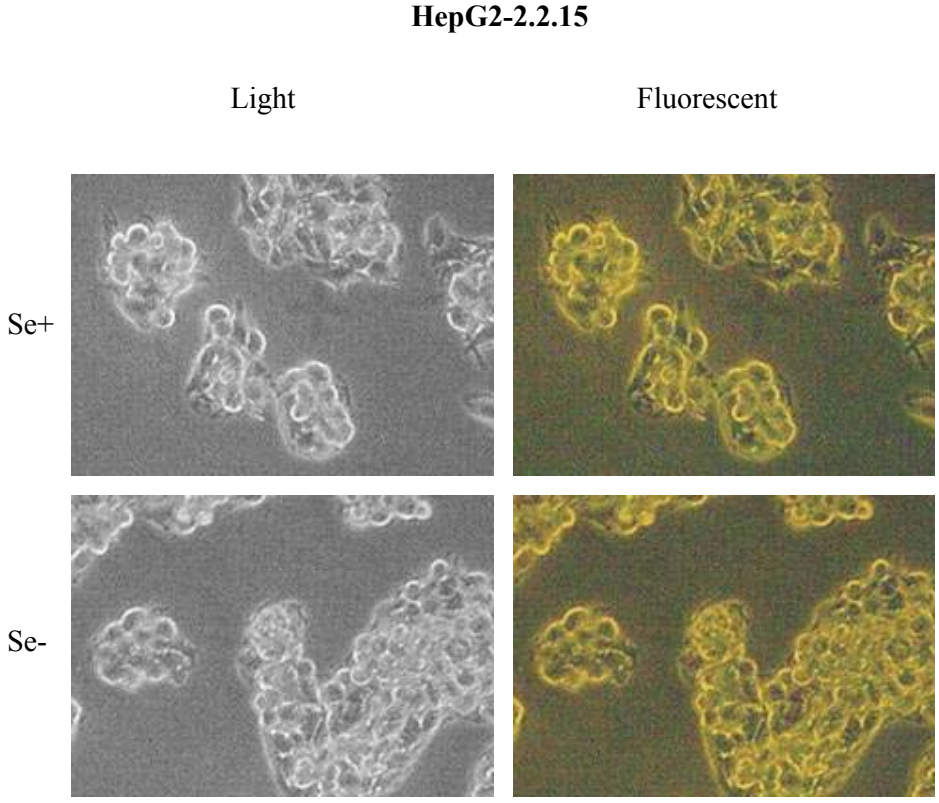

Figure 4.10.2 cont': Analysis of oxidative stress in isogenic cell lines by oxidant-sensitive fluorescent dye DCFH-DA in HAM's medium. HepG2-2.2.15 cells were cultured under selenium-adequate (Se+) and selenium-deficient (Se-) conditions for 96 hours. Absence of cells with fluorescence signal under selenium-deficient conditions indicates that HepG2-2.2.15 cells do not harbor oxidative stress (right panel). Light microscopy photographs (left panel) of the corresponding area of fluorescent photographs of HepG2-2.2.15 cells under selenium-adequate and selenium-deficient conditions were also shown. Original magnification 200×.

Following this, we decided to introduce increasing doses of H_2O_2 to the culture medium on the 3rd day of cultivation in DMEM under selenium-adequate and selenium-deficient conditions in order to observe the differential response, if exists, of these two isogenic cell lines to extreme controlled oxidative stress conditions under selenium-deficiency. To find this out, HepG2 and HepG2-2.2.15 cells were cultured under selenium-adequate and selenium-deficient conditions for 3 days, and treated with increasing doses of H_2O_2 for additional 4 hours. After that, HepG2 and HepG2-2.2.15 cells under oxidative stress and apoptosis were counted by DCFH assay and Hoechst 33258 nuclear staining, respectively.

The graphs in Figure 4.10.3 demonstrate that both cell lines tolerated excess oxidative stress under selenium-adequate conditions as expected since selenoenzyme, Gpx, activity under this condition should be intact. The difference in response to excess ROS was exhibited under selenium-deficient conditions. At the 3rd day of the culture under selenium-deficient conditions, in the absence of exogenous H_2O_2, about 18% of HepG2 cells were under oxidative stress cells. At that time, about 35% of HepG2 cells were apoptotic. As much as 25 μM H_2O_2 was enough to increase intracellular oxidative stress and accordingly apoptotic cells in HepG2 cells under selenium-deficient conditions. By addition of increasing doses of H_2O_2, HepG2 cells had gradual increase in the number of cells under oxidative stress that paralleled the increase in the number of the apoptotic cells. At 200 μM about 50% of HepG2 cells were under oxidative stress and about 70% was dead. HepG2-2.2.15 cells showed neither any sign of oxidative stress nor associated cell death even at the highest concentration of ROS.

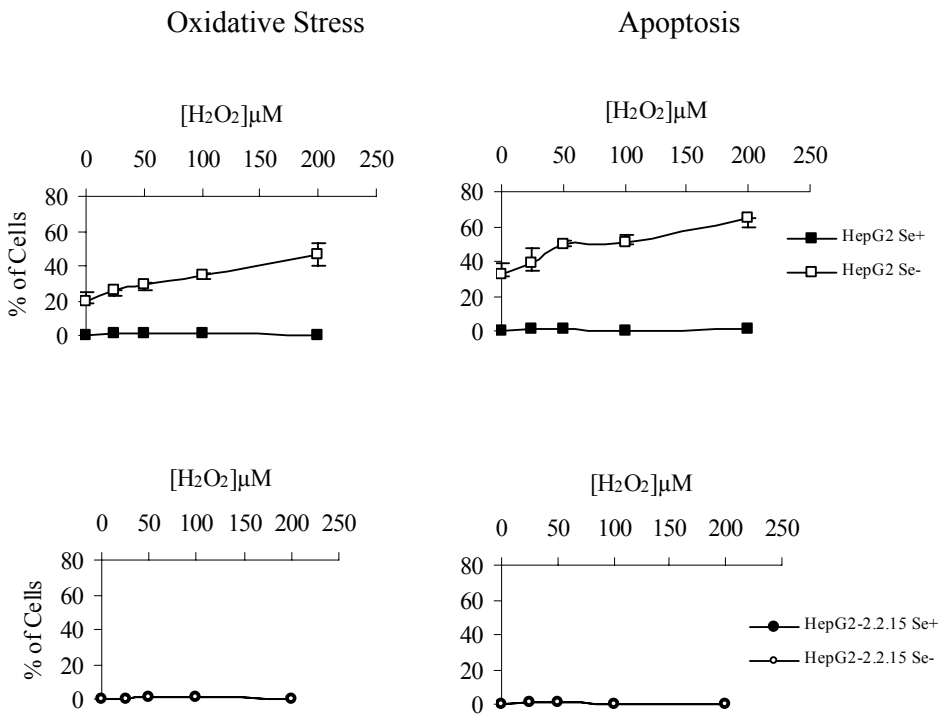

Figure 4.10.3: Correlation of oxidative stress with apoptosis in isogenic cell lines. HepG2 (upper panel) and HepG2-2.2.15 (lower panel) cells were cultured in selenium-adequate (Se+) and selenium-deficient (Se-) DMEM for 72 hours and treated with indicated concentrations of H_2O_2. Four hours later, cells that are under oxidative stress are counted by fluorescence microscope and apoptotic cells were counted by Hoechst 33258 staining. The results are from three separate experiments. Error bars indicate standard deviation.

Accordingly, HepG2 cells displayed apoptotic features under selenium-deficiency induced oxidative stress whereas HepG2-2.2.15 cells did not. Figure 4.10.4 shows NAPO immunostaining of HepG2 and HepG2-2.2.15 cells that were cultured in DMEM under selenium-adequate and selenium-deficient conditions on the 4th day. It is apparent from the figure that apoptotic selenium-deficient HepG2 cells display NAPO antigen, while non-apoptotic HepG2 cells under the same conditions do not. Most probably these cells will lose NAPO antigen soon. On the contrary, selenium-deficient HepG2-2.2.15 cells do not lose NAPO. In addition under selenium-supplementation, both cell lines are non-apoptotic and display NAPO immunostaining. Condensed Hoechst 33258 nuclear staining reveals the apoptotic cells in the same figure.

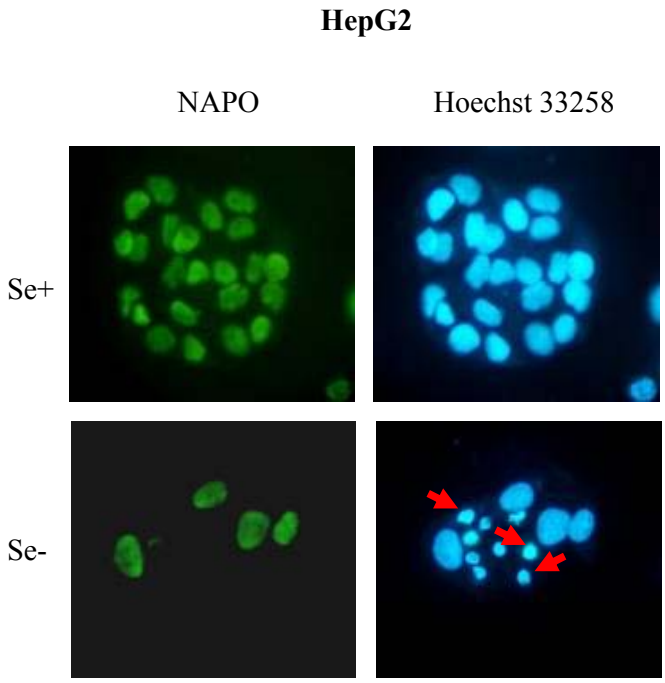

Figure 4.10.4: NAPO immunostaining of isogenic cell lines in DMEM. NAPO immunostaining of HepG2 cells. Cells were cultured in DMEM under selenium-adequate (Se+) and selenium-deficient (Se-) conditions. Left panel shows the NAPO immunostaining and right panel shows the nuclear Hoechst 33258 staining of the corresponding area of NAPO staining. Note that apoptotic HepG2 cells with condensed nuclei (red arrows) are positive for NAPO assay under selenium-deficient conditions.

HepG2-2.2.15

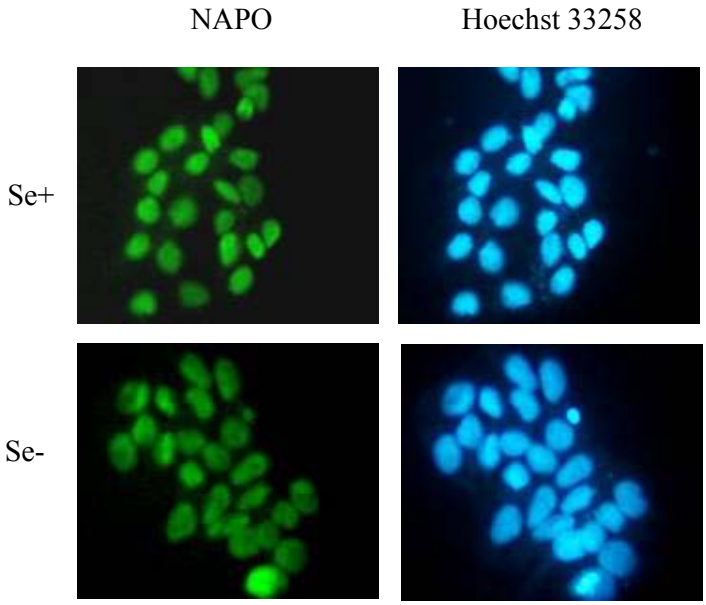

Figure 4.10.4 cont': NAPO immunostaining of isogenic cell lines in DMEM. NAPO immunostaining of HepG2-2.2.15 cells. Cells were cultured under selenium-adequate (Se+) and selenium-deficient (Se-) conditions. Left panel shows the NAPO immunostaining and right panel shows the nuclear Hoechst 33258 staining of the corresponding area of NAPO staining. Note that HepG2-2.2.15 cells are negative for NAPO assay under both conditions.

Moreover we applied the NAPO assay to HepG2 and HepG2-2.2.15 cells plated in selenium-adequate and selenium-deficient HAM's medium on the 4th day of the culture. As it is clearly depicted in Figure 4.10.5 that death HepG2 cells lose NAPO antigen under selenium-deficient conditions confirming that they die by apoptosis. On the contrary, live HepG2, and HepG2-2.2.15 cells under the same condition maintain NAPO antigen like both HepG2 and HepG2-2.2.15 cultured under selenium-adequate conditions.

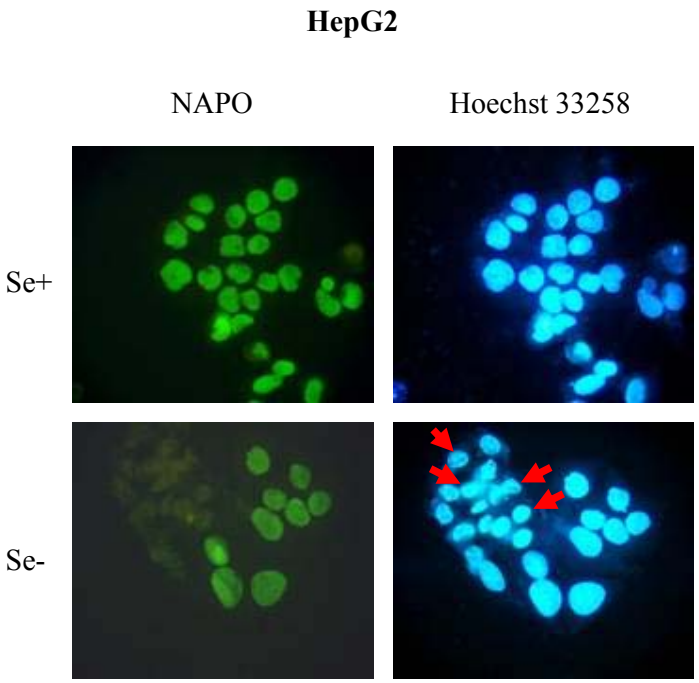

Figure 4.10.5: NAPO immunostaining of isogenic cell lines in HAM's medium. NAPO immunostaining of HepG2 cells. Cells were cultured under selenium-adequate (Se+) and selenium-deficient (Se-) conditions. Left panel shows the NAPO immunostaining and right panel shows the nuclear Hoechst 33258 staining of the corresponding area of NAPO staining. Note that apoptotic HepG2 cells with condensed nuclei (red arrows) are positive for NAPO assay under selenium-deficient conditions.

HepG2-2.2.15

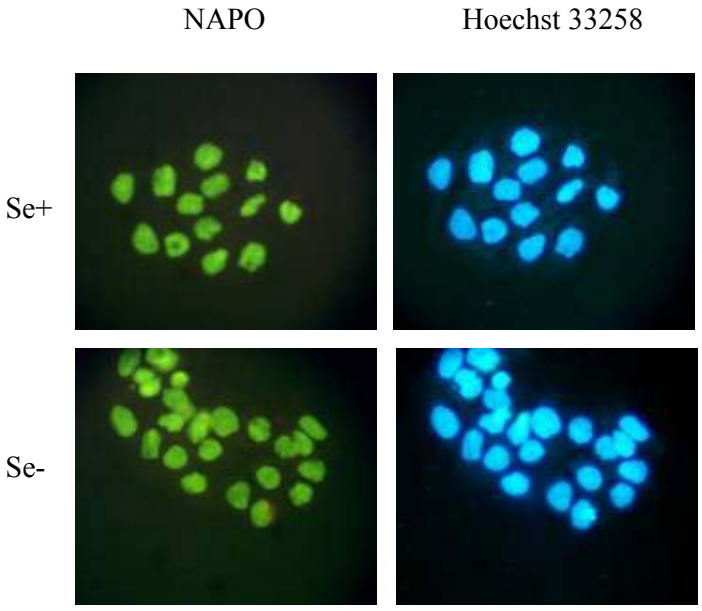

Figure 4.10.5 cont': NAPO immunostaining of isogenic cell lines in HAM's medium. NAPO immunostaining of HepG2-2.2.15 cells. Cells were cultured in DMEM under selenium-adequate (Se+) and selenium-deficient (Se-) conditions. Left panel shows the NAPO immunostaining and right panel shows the nuclear Hoechst 33258 staining of the corresponding area of NAPO staining. Note that HepG2-2.2.15 cells are negative for NAPO assay under both conditions.

Progressive accumulation of the cells under oxidative stress and subsequent increase in apoptotic cells were accompanied with the release of cytochrome c from intermembrane space of mitochondria to cytoplasm in selenium-deficient HepG2 cells on the 4th day of the culture in DMEM. Figure 4.10.6 clearly shows the diffuse and intense cytoplasmic cytochrome c immunostaining of apoptotic HepG2 cells under selenium-deficiency, and punctuate mitochodrial cytochrome c stain of HepG2 cells under selenium-adequate conditions. In contrast to HepG2 cells, cytochrome c of HepG2-2.2.15 cells were mitochondrial with punctuate stain conditions under both.

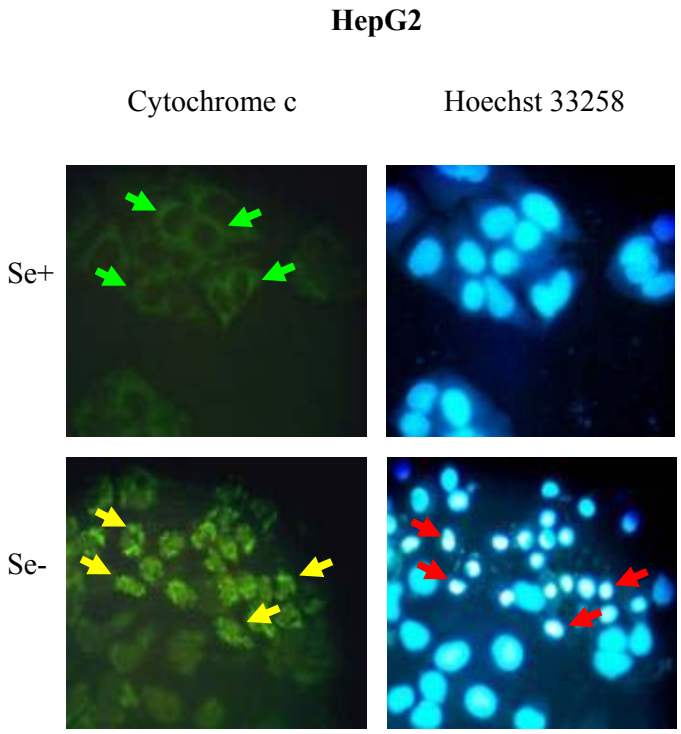

Figure 4.10.6: Cytochrome c immunostaining of isogenic cell lines cultured in DMEM. Cytochrome c immunostaining of HepG2 cells. Cells were cultured in DMEM under selenium-adequate (Se+) and selenium-deficient (Se-) conditions. Left panel shows cytochrome c immunostaining and right panel shows nuclear Hoechst 33258 staining of the corresponding area of cytochrome c staining. Note that HepG2 cells with condensed nuclei (red arrows) have diffuse and strong cytoplasmic cytochrome c immunostaining pattern (yellow arrows) under selenium-deficient conditions but not under selenium-adequate conditions (green arrows).

HepG2-2.2.15

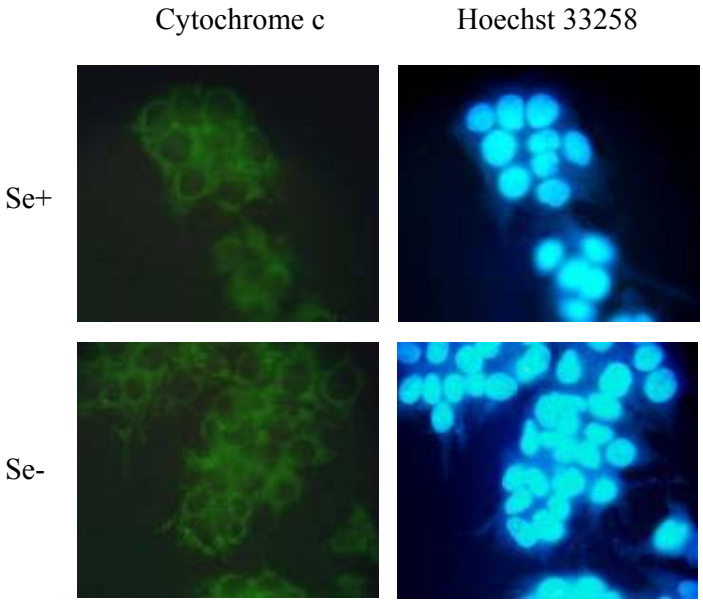

Figure 4.10.6.cont': Cytochrome c immunostaining of isogenic cell lines cultured in DMEM. Cytochrome c immunostaining of HepG2-2.2.15 cells. Cells were cultured in DMEM under selenium-adequate (Se+) and selenium-deficient (Se-) conditions. Left panel shows cytochrome c immunostaining and right panel shows nuclear Hoechst 33258 staining of the corresponding area of cytochrome c staining. Note that HepG2-2.2.15 cells have similar punctuate mitochondrial cytochrome c staining under both conditions.

Subsequent cleavage of PARP by active caspase 3 of selenium-deficient HepG2 cells that were cultured in DMEM until there was 90% apoptosis was demonstrated by immunoblotting in Figure 4.10.7. On the other hand, under the same conditions, HepG2-2.2.15 cells had intact PARP protein due to its inactive caspase 3. Under selenium-adequate conditions, both cell lines had intact PARP protein since they had mitochondrial cytochrome c and consequently inactive caspase 3. CK18 staining in Figure 4.10.7 confirms the specificity of the cleavage since equal levels of protein exists under both conditions.

As Huh-7 cells, HepG2 cells displayed cytochrome c release most probably due to the disruption of mitochondrial membrane by the accumulated ROS, and subsequent PARP cleavage due to its active caspase 3. On the contrary, most probably, HepG2-2.2.15 cells have acquired resistance to oxidative stress under selenium-deficiency like Hep3B-TR cells, and tolerated the stress condition most likely by the presence of a protective antioxidant mechanism that gives them survival advantage under selenium-deficiency.

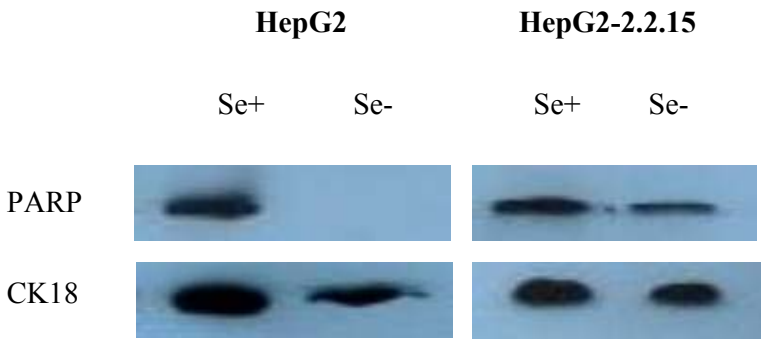

Figure 4.10.7: Western blot analysis of PARP protein in isogenic cell lines. HepG2 and HepG2-2.2.15 cells were cultured in DMEM under selenium-adequate (Se+) and selenium-deficient (Se-) conditions. Upper panel shows PARP immunoblotting, and the lower panel shows CK18 immunoblotting, which is used as an equal loading control. Note that HepG2 cells lose PARP protein under selenium-deficient condition, while HepG2-2.2.15 cells do not.

4.11 Response of Other Cell Lines to Selenium-Deficiency

Finally, we speculated the response of other cell lines to our selenium-deficient conditions, and extended our study to cell lines of other cancer types, including, breast, colon, and melanoma. Under our experimental conditions we tested a total of 27 different cancer cell lines that included 14 breast, 10 colon, and 3 melanoma. As shown in Table 4.11.1, among 14 breast cancer cell lines 11, 10 colon cancer cell lines 8, and 3 melanoma cell lines all were resistant to selenium-deficiency induced cell death. This preliminary data may suggest that escape from deadly consequences of selenium-deficiency may play a role in other cancer types. These cell lines have been reported previously; PMC-42 by Lauerova *et al.*, 1988; HBL-100, MCF-7, and MDA-MB-231 by Chandrasekaran and Davidson, 1979; HMT-3552 by Briand *et al.*, 1987; BT-474 by Lasfargues *et al.*, 1978; GI-101 by Hurst *et al.*, 1993; MDA-MB-175 by Rizk, 1982; CAL-51 by Chedin *et al.*, 1997; MDA-MB-468 by Reisbach *et al.*, 1982; T-47-D by Vic *et al.*, 1982; Sk-Br-5 by Rakowicz-Szulczynska *et al.*, 1992; ZR-75-1 by Engel *et al.*, 1978; CAMA-1 by Tsang *et al.*, 1981; SW480 and SW620 by Lelbovitz *et al.*, 1979; KM12 by Morikawa *et al.*, 1988; SW48 by Gaffney *et al.*, 1983; HT29 by von Kleist *et al.*, 1975; HCT15 by Budach *et al.*, 1993; TC7 by Mallo *et al.*, 1997; HCT-8 by Schultz *et al.*, 1975; LoVo by Drewinko *et al.*, 1976; and finally TC71 by Pocard *et al.*, 2000.

Table 4.11.1: Response of Other Cell Lines to Selenium-Deficiency.

Cell Lines	Cell Death
Breast	
PMC-42	No
HBL-100	No
HMT-3552	No
MCF-7	No
BT-474	No
GI-101	No
MDA-MB-175	No
CAL-51	No
MDA-MB-468	No
T-47-D	No
Sk-Br-5	No
ZR-75-1	Yes
MDA-MB-231	Yes
CAMA-1	Yes
Colon	
SW480	No
SW620	No
KM12	No
SW48	No
HT29	No
HCT15	No
TC7	No
HCT-8	No
LoVo	Yes
TC71	Yes
Melanoma	
WMI15	No
SkaNa12	No
SUEP-Propres	No

CHAPTER 5. DISCUSSION and PERSPECTIVES

Selenium is an essential dietary supplement for human health and the necessity of this trace element has been known for almost 50 years (Flohe *et al.*, 2000). Pancreatitis, loss of immunocompetence, reproductive deficiencies, and disorders related to thyroid hormone metabolism, viral infections, and cardiovascular diseases have been reported to be associated with low selenium status (Rayman, 2000). Besides, epidemiological studies have suggested an inverse relationship between selenium levels and different cancer cases including prostate cancer (Duffield-Lillico *et al.*, 2003; Yoshizawa *et al.*, 1998; Willett *et al.*, 1983), lung cancer (Knekt *et al.*, 1998; Knekt *et al.*, 1990), gastrointestinal cancer (Willett *et al.*, 1983), stomach cancer (Kneckt *et al.*, 1990), squamous esophageal and gastric cardia cancers (Mark *et al.*, 2000), and liver cancer (Corrocher *et al.*, 1986; Yu *et al.*, 1999; Buljevac *et al.*, 1996). However, regardless of the growing evidence related to its deficiency and certain disease conditions, the cellular changes in the absence of selenium and the mechanism of the protective effect of selenium are poorly elucidated.

The association of selenium-deficiency with hepatic disease conditions including hepatocellular carcinoma is well documented. Yet, the underlying molecular mechanisms of this association and the protection of selenium are poorly characterized. This research project has aimed to improve the current knowledge related to selenium-deficiency and the associated tumorogenesis of hepatocytes ending up with malignant behavior.

First, we wanted to develop an *in vitro* experimental selenium-deficient model using human HCC cell lines. It would be more advantages than *in vivo* models in terms of ease of perform, control of experimental parameters, and homogeneity of cell populations. Moreover, the results obtained with human cell lines rather than animal tissues would be more trustful and comparable in concluding the biological changes in human.

Our extensive observations with Huh-7 and HepG2 cell lines demonstrate that *in vitro* selenium-deficiency recapitulates the hepatocellular injury, observed in mice fed with selenium-deficient diet (Cheng *et al.*, 2003). Huh-7 and HepG2 cell lines act as 'hepatocyte like' malign cells since they undergo delayed apoptotic cell death following 3-4 days of culture under selenium-deficient conditions. This apoptotic type of cell death is associated with the loss of Gpx activity and correlated well with the presence of oxidative stress and the release of cytochrome c from mitochondria to cytoplasm. Under oxidative stress conditions, Gpx catalyzes the reduction of phospholipid hydroperoxides and hydrogen peroxide, acting as an anti-apoptotic factor (Nomura *et al.*, 2000).

The release of cytochrome c from electron transport chain, and finally from intermembrane space of mitochondria, where it is located, to the cytoplasm is illustrated to be triggered by H_2O_2 and to be associated with apoptotic response (Stridh *et al.*, 1998; Cai *et al.*, 1998; Morales *et al.*, 1997; Atlante *et al.*, 2000). Moreover, it has been previously reported that H_2O_2, which is produced by SOD enzymes, is converted to $OH^{\cdot}$ by Fenton reaction. In addition, it has been illustrated that the release of cytochrome c to cytoplasm was associated with the peroxidation of mitochondrion specific phospholipid, cardiolipin (Stridh *et al.*, 1998; Cai *et al.*, 1998; Morales *et al.*, 1997; Atlante *et al.*, 2000; Nomura *et al.*, 2000; Shidoji *et al.*, 1999). Moreover, Vitamin E influences the oxidative stress status of the cell. It has a protective scavenger effect against free radical damage. Scavenger role of this vitamin is achieved through integration in cellular membranes and reduction of $OH^{\cdot}$ downstream of selenium dependent Gpx and by this way protection of peroxidation of lipids (Claycombe and Meydani, 2001).

In our experiments performed with the inclusion of Vitamin E, we observed neither the oxidative stress nor the apoptosis in Huh7 cells under selenium-deficient conditions. Vitamin E had indifferent effect on Hep3B-TR cells under the same conditions since we did not observe any oxidative. These results suggest that in Huh-7 cells under selenium-deficiency induced oxidative stress conditions, the absence of a ROS scavenger like Vitamin E could cause the disruption of the integrity of mitochondrial membrane. Peroxidation of mitochondrial membrane phospholipids, such as cardiolipins due to uneliminated H_2O_2 and consequent $OH^{\cdot}$, could lead to the release of cytochrome c leading to apoptotic response of these cells. Our experimental data related to the gain of resistance of Huh-7 cells to selenium-deficiency induced oxidative stress and apoptosis in the presence of Vitamin E support this mechanism.

Cytoplasmic cytochrome c triggers an apoptotic response with the activation of downstream apoptotic molecules, including caspase 9 and caspase 3 as evidenced here by the degradation of PARP, which has been reported to be the specific known target of caspase 3 (Kaufmann *et al.*, 1993). The immediate trigger of apoptosis by the release of cytochrome c is also supported by our experiments such as, TUNEL, Annexin V, NAPO, and PARP immunoblotting. The apoptotic response can be considered as a cellular escape mechanism to avoid the oxidative stress induced damage to cellular biomolecules in hepatocytes and 'hepatocyte like' malign cells like Huh-7 and HepG2. On the other hand, this apoptotic response was independent of p53 since Huh-7 has a mutant p53 (Hsu *et al.*, 1993). On the contrary, with selenium supplementation, we have observed a recovery in these cells, with the gain of Gpx activity, absence of oxidative stress, the mitochondrial localization of cytochrome c, and finally the prevention of apoptotic cell death. Under selenium-adequate conditions, antioxidant selenium-dependent enzyme, Gpx, is active and reduces H_2O_2 before it is converted to $OH^{\cdot}$ by the Fenton reaction.

Next, to our surprise most of HCC cell lines that we studied (10/13; 77%) demonstrated a tolerant behavior under selenium-deficient conditions with the absence of oxidative stress and associated lack of apoptotic response. Tumor cells in this group had a great survival and proliferative capacity for at least 10 days

under selenium-deficient conditions. The detailed examination of Hep3B-TR cells that is among the 77%, clearly demonstrated that these malign cells could counteract the extensive oxidative stress. This was evidenced by the tolerant response of these malign hepatocytes to oxidative stress challenge introduced by treating these cells with increasing doses of H_2O_2 with the absence of oxidative stress as illustrated by the DCFH assay. As expected, they have mitochondrially localized cytochrome c, and intact PARP under selenium-deficiency showing that they do not undergo apoptosis, which is in complete contradiction to 'hepatocyte like' malign cells which constitute the remaining 23%. Most probably, selenium-deficiency tolerant malign cells could have been adapted so that they can counteract the ROS generated by their respiratory chain as a physiological metabolic activity. The fact that Huh-7 and HepG2 cell lines have gained resistance to selenium-deficiency induced oxidative stress and apoptosis in the presence of Vitamin E suggests that oxidative stress and apoptosis resistant cell lines could have adapted a mechanism to scavenge ROS produced by electron transport chain in the absence of selenium. Nevertheless, this adaptation cannot be explained by the selenium reservoir of the resistant cells due to the slow rate of proliferation since the proliferation capacity of these cells is the same under selenium-adequate and selenium-deficient conditions.

It has been well established that selenium is an essential dietary nutrient for animals and men. Selenium-deficiency is associated with many disease conditions. It would be highly unlikely to observe a normal cell showing tolerance to its deficiency. Our extensive experiments with HBV genome transfected HepG2-2.2.15 cell line also demonstrated that the capability of showing a tolerant response to selenium-deficiency induced oxidative stress and related apoptosis is acquired by the highly transformed malignant hepatocytes. Moreover, it is very interesting to detect that this acquired capability was associated with the presence of HBV sequences in the genomes of these tolerant malign cells.

These malignant cells could have gained this tolerance to selenium-deficiency induced oxidative stress *in vitro* or *in vivo*. HBV integration is detected in 9 of the 10 tolerant cell lines (Table 4.8.1). The remaining cell line, Mahlavu, exhibits an aflatoxin-related p53 mutation (Bressac *et al.*, 1991; Hsu *et al.*, 1991). It is

documented that chronic HBV infection and aflatoxin exposure cause generation of ROS in hepatocytes (Yang *et al.*, 2000; Shen *et al.*, 1995; Shen *et al.*, 1996). Oxidative stress is regarded as an important player in hepatocyte malignancy (Toyokuni *et al.*, 1995). Depending on these available data and literature, it will not be unlikely to consider that the acquired tolerance to selenium-deficiency could be gained *in vivo*.

Meanwhile, we inspected the molecular mechanism that relies under this acquired tolerance to selenium-deficiency. In order to investigate this we examined Akt protein, which is important in regulating cell survival and apoptosis and influences cell survival upon oxidative injury (Ostrakhovitch *et al.*, 2002), under selenium-deficient or supplemented conditions with Huh-7 and Hep3B-TR cells. In our preliminary results, we did not observe any difference in un-phosphorylated or active phosphorylated Akt protein levels in apoptosis sensitive (Huh-7) and apoptosis resistant (Hep3B-TR) cell lines. Moreover, our preliminary data with similar levels of Akt protein in Hep3B-TR cells under selenium-deficient and selenium supplemented conditions supports our results related to the lack of oxidative stress in this cell line under selenium-deficiency. A very recent paper illustrated the anti-apoptotic effect of selenium following selenite supplementation to Huh-7 cells that had been cultured in serum free medium. In parallel, they illustrated the activation PI3K/Akt kinase pathway and inactivation of ASK1 as the changes that prevent apoptosis and induce cull survival in these cells. Moreover, the activation of FAK and Rac1, upstream inducers of PI3K, have been suggested to have a role in the activation of PI3K pathway in this study. The experiments in this study has been performed in a comparable way with Huh-7 cells that had been cultured in DMEM with 10% FBS, serum free medium that contained 0.1% FBS, and selenium supplemented serum free medium (Lee *et al.*, 2003). Based on these results, we consider the investigation of PI3K/Akt kinase pathway for further studies.

Finally we wanted to extend our study to other cancer cell lines including breast, colon, and melanoma. We have analyzed a total of 27 more cancer cell lines, 14 being breast, 10 colon, and 3 melanoma. Eleven of the 14 breast cancer cell lines 11, eight of 10 colon cancer cell lines, and all of the 3 melanoma cell lines were

resistant to selenium-deficiency induced cell death. Our preliminary data may suggest that escape from deadly consequences of selenium-deficiency may play a role in other cancer types. In addition dietary selenium supplementation can be an invaluable approach in terms of the treatment of cancer patients.

This tolerance of most HCC cell lines to oxidative stress is only observed under selenium-deficient conditions, and this phenotype is not expressed under selenium-adequate conditions. So, selenium-deficiency provides these malign cells a cryptic survival advantage that is hidden under selenium-adequate conditions.

The experiments can be extended to *in vivo* animal models of different cancer types to compare with the *in vitro* responses that we have obtained with the cell lines we have studied. Finally, the molecular mechanisms and the exact signaling pathways leading to this tolerant behavior of the cancer cells can be further investigated in a comparable way. In addition to this, the selenium-deficiency induced oxidative stress sensitive and resistant cell lines can be subjected to large scale genome analysis under selenium-adequate and selenium-deficient conditions.

So far, we believe that we have brought a new insight to scientific debate related to the role of selenium-deficiency in giving survival advantage to malignant hepatocytes and providing a mechanism that ends up with hepatocarcinoma. Scientists all over the world working with selenium are aware of the importance of selenium in human health. The PRECISE (Prevention of Cancer by Intervention with Selenium) study is planning to recruit about 33,000 European to participate in a study on the effect of selenium on mood and quality of life (http://www.surrey.ac.uk; http://janis7hepc.com/Nutrition/Selenium.htm;). In addition, The United States National Cancer Institute has agreed to fund a 12-year study. Furthermore, SELECT (Selenium and Vitamin E Cancer Prevention Trial) decided to recruit 32,000 men to participate in the study on the effect of selenium and vitamin E on prostate cancer (Klein, 2003).

Benefits of dietary selenium supplementation to cancer patients is under debate. With our study which was mostly based on HCC cell lines, we think that we

brought a new approach on the scientific discussions about selenium supplementation to cancer patients. Our data with breast, colon, and melanoma cell lines encourage further investigation relating selenium supplementation to these patients.

WEB SOURCES

http://www.surrey.ac.uk.

http://www.roche-applied-science.com.

http://janis7hepc.com/Nutrition/Selenium.htm.

REFERENCES

Achenbach, T. V., Muller, R., and Slater, E. P. Bcl-2 independence of flavopiridol-induced apoptosis. Mitochondrial depolarization in the absence of cytochrome c release. (2000). *J. Biol. Chem.*, **275**, 32089-32097.

Ames, B. N. (1983). Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. *Science*, **221**, 1256-1264.

Amundson, S. A., Myers, T. G., and Fornace, A. J. Jr. (1998). Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress. *Oncogene*, **17**, 3287-3299.

Anane, R., andCreppy, E. E. (2001). Lipid peroxidation as pathway of aluminium cytotoxicity in human skin fibroblast cultures: prevention by superoxide dismutase+catalase and vitamins E and C. *Hum. Exp. Toxicol.*, **20**, 477-481.

Arthur, J. R. (2000). The glutathione peroxidases. *Cell. Mol. Life Sci.*, **57**, 1825-1835.

Arthur, J. R., Bermano, G., Mitchell, J. H., and Hesketh, J. E. (1996). Regulation of selenoprotein gene expression and thyroid hormone metabolism. *Biochem. Soc. Trans.*, **24**, 384-388.

Atadja, P., Wong, H., Garkavtsev, I., Veillette, C., and Riabowol, K. (1995). Increased activity of p53 in senescing fibroblasts. *Proc. Natl. Acad. Sci. U. S. A* **92**, 8348-8352.

Atlante, A., Calissano, P., Bobba, A., Azzariti, A., Marra, E., and Passarella, S. (2000). Cytochrome c is released from mitochondria in a reactive oxygen species (ROS)-dependent fashion and can operate as a ROS scavenger and as a respiratory substrate in cerebellar neurons undergoing excitotoxic death. *J. Biol. Chem.*, **275**, 37159-37166.

Aust, S. D., Roerig, D. L., Pederson, T. C. (1972). Evidence for superoxide generation by NADPH-cytochrome c reductase of rat liver microsomes. *Biochem. Biophys. Res. Commun.*, **47**, 1133-1137.

Baker, R. D., Baker, S. S., LaRosa, K., Whitney, C., and Newburger, P. E. (1993). Selenium regulation of glutathione peroxidase in human hepatoma cell line Hep3B. *Arch. Biochem. Biophys.*, **304**, 53-57.

Barnes, D., and Sato, G. (1979). Growth of a human mammary tumour cell line in a serum-free medium. *Nature*, **281**, 388-389.

Beck, M. A., Shi, Q., Morris, V. C., and Levander, O. A. (1995). Rapid genomic evolution of a non-virulent coxsackievirus B3 in selenium-deficient mice results in selection of identical virulent isolates. *Nat. Med.*, **1**, 433-436.

Behne, D., Weiler, H., and Kyriakopoulos, A. (1996). Effects of selenium-deficiency on testicular morphology and function in rats. *J. Reprod. Fertil.*, **106**, 291-297.

Bendich, A. (2001). Micronutrients in women's health and immune function. *Nutrition*, **17**, 858-867.

Benn, J., and Schneider, R. J. (1994). Hepatitis B virus HBx protein activates Ras-GTP complex formation and establishes a Ras, Raf, MAP kinase signaling cascade. *Proc. Natl. Acad. Sci. U. S. A.*, **91**, 10350-10354.

Berger, M. M., Reymond, M. J., Shenkin, A., Rey, F., Wardle, C., Cayeux, C., Schindler, C., and Chiolero, R. L. (2001). Influence of selenium supplements on the post-traumatic alterations of the thyroid axis: a placebo-controlled trial. *Intensive Care Med.*, **27**, 91-100.

Berliner, J. A., and Heinecke, J. W. (1996). The role of oxidized lipoproteins in atherogenesis. *Free. Radic. Biol. Med.*, **20**, 707-727.

Bermano, G., Arthur, J. R., and Hesketh, J. E. (1996). Role of the 3' untranslated region in the regulation of cytosolic glutathione peroxidase and phospholipid-hydroperoxide glutathione peroxidase gene expression by selenium supply. *Biochem. J.*, **320**, 891-895.

Bermano, G., Nicol, F., Dyer, J. A., Sunde, R. A., Beckett, G. J., Arthur, J. R., and Hesketh, J. E. (1995). Tissue-specific regulation of selenoenzyme gene expression during selenium-deficiency in rats. *Biochem. J.*, **311**, 425-430.

Bhaskaram, P. (2002). Micronutrient malnutrition, infection, and immunity: an overview. *Nutr. Rev.*, **60**, 40-45.

Blagosklonny, M. V. (2002). P53: a ubiquitous target of anticancer drugs. *Int. J. Cancer*, **98**, 161-166.

Bock, A., Forchhammer, K., Heider, J., and Baron, C. (1991). Selenoprotein synthesis: an expansion of the genetic code. *Trends Biochem. Sci.*, **16**, 463-467.

Bosch, F. X., Ribes, J., and Borras, J. (1999). Epidemiology of primary liver cancer. *Semin. Liver Dis.*, **19**, 271-285.

Bradford, M. M. (1976).A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248-254.

Bressac, B., Kew, M., Wands, J., and Öztürk, M. (1991). Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature*, **350**, 429-431.

Briand, P., Petersen, O. W., and Van-Deurs, B. (1987). A new diploid nontumorigenic human breast epithelial cell line isolated and propagated in chemically defined medium. *In Vitro Cell. Dev. Biol.,* **23**, 181-188.

Britton, R. S. (1996). Metal-induced hepatotoxicity. *Semin. Liver Dis.*, **16**, 3-12.

Budach, W., Taghian, A., Freeman, J., Gioioso, D., and Suit, H. D. (1993). Impact of stromal sensitivity on radiation response of tumors. *J Natl Cancer Inst.*, **85**, 988-993.

Bulavin, D. V., Saito, S., Hollander, M. C., Sakaguchi, K., Anderson, C. W., Appella, E., and Fornace, A. J. Jr. (1999). Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. *EMBO J.*, **18**, 6845-6854.

Bulger, E. M., and Maier, R. V. (2003). An argument for Vitamin E supplementation in the management of systemic inflammatory response syndrome. *Shock*, **19**, 99-103.

Buljevac, M., Romic, Z., Vucelic, B., Banic, M., Krznaric, Z., and Plesko, S. (1996). Serum selenium concentration in patients with liver cirrhosis and hepatocellular carcinoma. *Acta Med. Croatica.*, **50**, 11-14.

Burgess, S., Geddes, J., Hawton, K., Townsend, E., Jamison, K., and Goodwin, G. (2002). Lithium for maintenance treatment of mood disorders. *Evid. Based Ment. Health*, **5**, 44.

Burk, R. F., Hill, K. E., and Motley, A. K. (2003). Selenoprotein metabolism and function: evidence for more than one function for selenoprotein P. *J. Nutr.*, **133**, 1517S-1520S.

Butts, B. D., Kwei, K. A., Bowden, G. .T, and Briehl, M. M. (2003). Elevated basal reactive oxygen species and phospho-Akt in murine keratinocytes resistant to ultraviolet B-induced apoptosis. *Mol. Carcinog.*, **37**, 149-57.

Cadet, J., Delatour, T., Douki, T., Gasparutto, D., Pouget, J. P., Ravanat, J. L., and Sauvaigo, S. (1999). Hydroxyl radicals and DNA base damage. *Mutat. Res.*, **424**, 9-21.

Cai, J., Yang, J., and Jones, D. P. (1998). Mitochondrial control of apoptosis: the role of cytochrome c. *Biochim. Biophys. Acta.*, **1366**, 139-149.

Camakaris, J., Voskoboinik, I., Mercer, J. F. (1999). Molecular mechanisms of copper homeostasis. *Biochem. Biophys. Res. Commun.*, **261**, 225-232.

Capdevila, J., Parkhill, L., Chacos, N., Okita, R., Masters, B. S., and Estabrook, R.W. (1981). The oxidative metabolism of arachidonic acid by purified cytochromes P-450. *Biochem. Biophys. Res. Commun.*, **101**, 1357-1363.

Castillo-Duran, C., and Uauy, R. (1988). Copper deficiency impairs growth of infants recovering from malnutrition. *Am. J. Clin. Nutr.*, **47**, 710-714.

Chambers, I., Frampton, J., Goldfarb, P., Affara, N., McBain, W., and Harrison, P. R. (1986). The structure of the mouse glutathione peroxidase gene: the selenocysteine in the active site is encoded by the 'termination' codon, TGA. *EMBO J.*, **5**, 1221-1227.

Chandra, J., Samali, A., and Orrenius, S. (2000). Triggering and modulation of apoptosis by oxidative stress. *Free. Radic. Biol. Med.*, **29**, 323-333.

Chandrasekaran, E. V., and Davidson, E. A. (1979). Glycosaminoglycans of normal and malignant cultured human mammary cells. *Cancer Res.*, **39**, 870-880.

Chariot, P., and Bignani, O. (2003). Skeletal muscle disorders associated with selenium-deficiency in humans. *Muscle Nerve.*, **27**, 662-668.

Chedin, M., Filhol, O., Duminy, C., Bolla, M., Benistant, C., Roche, S., Chambaz, E. M., and Cochet, C. (1997). Characterization of two different cytoplasmic protein tyrosine kinases from human breast cancer. *Carcinogenesis*, **18**, 1463-1472.

Cheeseman, K. H. (1993). Mechanisms and effects of lipid peroxidation. *Mol. Aspects Med.*, **14**, 191-197.

Cheng, W. H., Ho, Y. S., Valentine, B. A., Ross, D. A., Combs, G. F. Jr., and Lei, X. G. (1998). Cellular glutathione peroxidase is the mediator of body selenium to protect against paraquat lethality in transgenic mice. *J. Nutr.*, **128**, 1070-1076.

Cheng, W. H., Zheng, X., Quimby, F. R., Roneker, C. A., and Lei, X. G. (2003). Low levels of glutathione peroxidase 1 activity in selenium-deficient mouse liver affect c-Jun N-terminal kinase activation and p53 phosphorylation on Ser-15 in pro-oxidant-induced aponecrosis. *Biochem. J.*, **370**, 927-934.

Christensen, M. J., and Burgener, K. W. (1992). Dietary selenium stabilizes glutathione peroxidase mRNA in rat liver. *J. Nutr.*, **122**, 1620-1626.

Chu, F. F., and Esworthy, R. S. (1995). The expression of an intestinal form of glutathione peroxidase (GSHPx-GI) in rat intestinal epithelium. *Arch. Biochem. Biophys.*, **323**, 288-294.

Chu, F. F., Doroshow, J. H., and Esworthy, R. S. (1993). Expression, characterization, and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI. *J. Biol. Chem.*, **268**, 2571-2576.

Chu, F. F., Esworthy, R. S., Doroshow, J. H., Doan, K., and Liu, X. F. (1992). Expression of plasma glutathione peroxidase in human liver in addition to kidney, heart, lung, and breast in humans and rodents. *Blood*, **79**, 3233-3238.

Chung, M. H., Kasai, H., Jones, D. S., Inoue, H., Ishikawa, H., Ohtsuka, E., and Nishimura, S. (1991). An endonuclease activity of Escherichia coli that specifically removes 8-hydroxyguanine residues from DNA. *Mutat. Res.*, **254**, 1-12.

Claycombe, K. J., and Meydani, S. N. (2001). Vitamin E and genome stability. *Mutat. Res.*, **475**, 37-44.

Cohen, G., Yakushin, S., and Dembiec-Cohen, D. (1998). Protein L-dopa as an index of hydroxyl radical attack on protein tyrosine. *Anal. Biochem.*, **263**, 232-239.

Comstock, G. W., Burke, A. E., Hoffman, S. C., Helzlsouer, K. J., Bendich, A., Masi, A. T., Norkus, E. P., Malamet, R. L., and Gershwin, M. E. (1997). Serum concentrations of alpha tocopherol, beta carotene, and retinol preceding the diagnosis of rheumatoid arthritis and systemic lupus erythematosus. *Ann. Rheum. Dis.*, **56**, 323-325.

Cooke, M. S., Evans, M. D., Dizdaroglu, M., and Lunec, J. (2003). Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J.*, **17**, 1195-1214.

Corrocher, R., Casaril, M., Bellisola, G., Gabrielli, G., Hulpe, M., Garofoli, and E., Nicoli, N. (1986). Reduction of liver glutathione peroxidase activity and deficiency of serum selenium in patients with hepatocellular carcinoma. *Tumori.*, **72**, 617-619.

Czuczejko, J., Halota, W., Zachara, B. A., and Staubach-Topczewska, E. (2002). Plasma selenium concentration, glutathione peroxidase and glutathione S-transferase activities in patients with chronic liver diseases. *Pol. Merkuriusz Lek.*, **13**, 312-315.

Dandona, P., Thusu, K., Cook, S., Snyder, B., Makowski, J., Armstrong, D., and Nicotera T. (1996). Oxidative damage to DNA in diabetes mellitus. *Lancet*, **347**, 444-445.

De Haan, J. B., Bladier, C., Griffiths, P., Kelner, M., O'Shea, R. D., Cheung, N. S., Bronson, R. T., Silvestro, M. J., Wild, S., Zheng, S. S., Beart, P. M., Hertzog, P. J., and Kola, I. (1998). Mice with a homozygous null mutation for the most abundant glutathione peroxidase, Gpx1, show increased susceptibility to the oxidative stress-inducing agents paraquat and hydrogen peroxide. *J. Biol. Chem.*, **273**, 22528-22536.

De Maria, N., Colantoni, A., Fagiuoli, S., Liu, G. J., Rogers, B. K., Farinati, F., Van Thiel, D. H., and Floyd, R. A. (1996). Association between reactive oxygen species and disease activity in chronic hepatitis C. *Free Radic. Biol. Med.*, **21**, 291-295.

De Souza, A. T., Hankins, G. R., Washington, M. K., Orton, T. C., and Jirtle, R. L. (1995). M6P/IGF2R gene is mutated in human hepatocellular carcinomas with loss of heterozygosity. *Nat. Genet.* **11**, 447-449.

Dejean. A., and de The., H. (1990). Hepatitis B virus as an insertional mutagene in a human hepatocellular carcinoma. *Mol. Biol. Med.*, **7**, 213-222.

Diplock, A. T. (1994). Antioxidants and disease prevention. *Mol. Aspects Med.*, **15**, 293-376.

Dizdaroglu, M. (1992). Oxidative damage to DNA in mammalian chromatin. *Mutat. Res.*, **275**, 331-342.

Domigan, N. M., Charlton, T. S., Duncan, M. W., Winterbourn, C. C., and Kettle, A. J. (1995). Chlorination of tyrosyl residues in peptides by myeloperoxidase and human neutrophils. *J. Biol. Chem.*, **270**, 16542-16548.

Doroshow, J. H. (1995). Glutathione peroxidase and oxidative stress. *Toxicol. Lett.*, **82-83**, 395-398.

Draper, H. H., and Hadley, M. (1990). A review of recent studies on the metabolism of exogenous and endogenous malondialdehyde. *Xenobiotica*, **20**, 901-907.

Drewinko, B., Romsdahl, M. M., Yang, L. Y., Ahearn, M. J., and Trujillo, J. M. (1976). Establishment of a human carcinoembryonic antigen-producing colon adenocarcinoma cell line. *Cancer Res.*, **36**, 467-475.

Drisko, J. A., Chapman, J., and Hunter, V. J. (2003). The use of antioxidant therapies during chemotherapy. *Gynecol. Oncol.*, **88**, 434-439.

Dubourdeau, M., Miyamura, T., Matsuura, Y., Alric, L., Pipy, B., and Rousseau, D. (2002). Infection of HepG2 cells with recombinant adenovirus encoding the HCV core protein induces p21(WAF1) down-regulation - effect of transforming growth factor beta. *J. Hepatol.*, **37**, 486.

Duffield-Lillico, A. J., Dalkin, B. L., Reid, M. E., Turnbull, B. W., Slate, E. H., Jacobs, E. T., Marshall, J. R., and Clark, L. C. (2003). Selenium supplementation, baseline plasma selenium status and incidence of prostate cancer: an analysis of the complete treatment period of the Nutritional Prevention of *Cancer Trial. B. J. U. Int.*, **91**, 608-612.

Duggan, C., Gannon, J., and Walker, W. A. (2002). Protective nutrients and functional foods for the gastrointestinal tract. *Am. J. Clin. Nutr.*, **75**, 789-808.

Engel, L. W., Young, N. A., Tralka, T. S., Lippman, M. E., O'Brien, S. J., and Joyce, M. J. (1978). Establishment and characterization of three new continuous cell lines derived from human breast carcinomas. *Cancer Res.*, **38**, 3352-3364.

Enghild, J. J., Thogersen, I. B., Oury, T. D., Valnickova, Z., Hojrup, P., and Crapo, J. D. (1999). The heparin-binding domain of extracellular superoxide dismutase is proteolytically processed intracellularly during biosynthesis. *J. Biol. Chem.*, **274**, 14818-14822.

Enomoto, A., Esumi, M., Yamashita, K., Takagi, K., Takano, S., and Iwai, S. (2001). Abnormal nucleotide repeat sequence in the TGF-betaRII gene in hepatocellular carcinoma and in uninvolved liver tissue. *J. Patho.*, **195**, 349-354.

Esterbauer, H., Dieber-Rotheneder, M., Waeg, G., Striegl, G., and Jurgens, G., (1990). Biochemical, structural, and functional properties of oxidized low-density lipoprotein. *Chem. Res. Toxicol.*, **3**, 77-92.

Esworthy, R. S., Ho, Y. S., and Chu, F. F. (1997). The Gpx1 gene encodes mitochondrial glutathione peroxidase in the mouse liver. *Arch. Biochem. Biophys.*, **340**, 59-63.

Fariss, M. W., and Zhang, J. G. (2003). Vitamin E therapy in Parkinson's disease. *Toxicology*, **189**, 129-146.

Fernandez-Checa, J. C., Garcia-Ruiz, C., Colell, A., Morales, A., Mari, M., Miranda, M., Ardite, E. (1998). Oxidative stress: role of mitochondria and protection by glutathione. *Biofactors*, **8**, 7-11.

Flohe, L., Andreesen, J. R., Brigelius-Flohe, R., Maiorino, M., and Ursini, F. (2000). Selenium, the element of the moon, in life on earth. *IUBMB Life*, **49**: 411-420.

Floyd, R. A. (1990). The role of 8-hydroxyguanine in carcinogenesis. *Carcinogenesis*, **11**, 1447-1450.

Fong, T. L., Shindo, M., Feinstone, S. M., Hoofnagle, J. H., and Di Bisceglie, A. M. (1991). Detection of replicative intermediates of hepatitis C viral RNA in liver and serum of patients with chronic hepatitis C. *J. Clin. Invest.*, **88**, 1058-1060.

Fraga, C. G, Shigenaga, M. K., Park, J.W., Degan, P., and Ames, B. N. (1990). Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. *Proc. Natl. Acad. Sci. U. S. A.*, **87**, 4533-4537.

Freeman, B. A., Crapo, J. D. (1982). Biology of disease: free radicals and tissue injury. *Lab. Invest.*, **47**, 412-426.

Fu. Y., Cheng, W. H., Porres, J. M., Ross, D. A., and Lei, X. G. (1999). Knockout of cellular glutathione peroxidase gene renders mice susceptible to diquat-induced oxidative stress. *Free Radic. Biol. Med.*, **27**, 605-611.

Fuchs, S. Y., Adler, V., Pincus, M. R., and Ronai, Z. (1998). MEKK1/JNK signaling stabilizes and activates p53. *Proc. Natl. Acad Sci. USA.*, **95**, 10541-10546.

Gaetke, L. M., and Chow, C. K. (2003). Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology*, **189**, 147-163.

Gaffney, E. V., Tsai, S. C., Dell'Aquila, M. L., and Lingenfelter, S. E. (1983). Production of growth-inhibitory activity in serum-free medium by human monocytic leukemia cells. *Cancer Res.*, **43**, 3668-3673.

Galle, J., Bengen, J., Schollmeyer, P., and Wanner, C. (1995). Impairment of endothelium-dependent dilation in rabbit renal arteries by oxidized lipoprotein(a). Role of oxygen-derived radicals. *Circulation*, **92**, 1582-1589.

Gartner, R., Gasnier, B. C., Dietrich, J. W., Krebs, B., and Angstwurm, M. W. (2002). Selenium supplementation in patients with autoimmune thyroiditis decreases thyroid peroxidase antibodies concentrations. *J. Clin. Endocrinol. Metab.*, **87**, 1687-1691.

Gate, L., Paul, J., Ba, G. N., Tew, K. D., and Tapiero, H. (1999). Oxidative stress induced in pathologies: the role of antioxidants. *Biomed. Pharmacother.*, **53**, 169-180.

Geiger, P. G., Thomas, J.P., and Girotti, A. W. (1991). Lethal damage to murine L1210 cells by exogenous lipid hydroperoxides: protective role of glutathione-dependent selenoperoxidases. *Arch. Biochem. Biophys.*, **288**, 671-680.

Gera, T., and Sachdev, H. P. (2002). Effect of iron supplementation on incidence of infectious illness in children: systematic review. *B. M. J.*, **325**, 1142.

Gerber, M. A., Shieh, Y. S., Shim, K.S., Thung, S. N., Demetris, A. J., Schwartz, M., Akyol, G., and Dash, S. (1992). . Detection of replicative hepatitis C virus sequences in hepatocellular carcinoma. *Am. J. Pathol.*, **141**, 1271-1277.

Graef, E., Caselmann, W. H., Wells, J., and Koshy, R. (1994). Insertional activation of mevalonate kinase by hepatitis B virus DNA in a human hepatoma cell line. *Oncogene*, **9**, 81-87.

Guan, Y., Hickey, M. J., Borgstahl, G. E., Hallewell, R. A., Lepock, J. R., O'Connor, D., Hsieh, Y., Nick, H. S., Silverman, D. N., and Tainer, J. A. (1998). Crystal structure of Y34F mutant human mitochondrial manganese superoxide dismutase and the functional role of tyrosine 34. *Biochemistry*, **37**, 4722-4730.

Guarini, P., Stanzial, A. M., Olivieri, O., Casaril, M., Galvani, S., Pantalena, M., and Corrocher, R. (1998). Erythrocyte membrane lipids and serum selenium in post-viral and alcoholic cirrhosis. *Clin. Chim. Acta.*, **270**, 139-150.

Hagen, T. M., Huang, S., Curnutte, J., Fowler, P., Martinez, V., Wehr, C. M., Ames, B. N., and Chisari, F. V. (1994). Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 12808-12812.

Halliwell, B. and Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*, **3rd edition.**

Haratake, J., Horie, A., and Takeda, S. (1987).Histochemical and ultrastructural study of copper-binding protein in hepatocellular carcinoma. *Cancer*, **60**, 1269-1274.

Hasegawa, K., Wang, Z., Inagaki, M., and Carr, B. I. (1995). Characterization of a human hepatoma cell line with acquired resistance to growth inhibition by transforming growth factor beta 1 (TGF-beta 1). *In Vitro Cell Dev. Biol. Anim.*, **31**, 55-61.

Helmy, M. H., Ismail, S. S., Fayed, H., and El-Bassiouni, E. A. (2000). Effect of selenium supplementation on the activities of glutathione metabolizing enzymes in human hepatoma Hep G2 cell line. *Toxicology*, **144**, 57-61.

Henkler, F. F., and Koshy, R. (1996). Hepatitis B virus transcriptional activators: mechanisms and possible role in oncogenesis. *J. Viral. Hepat.*, **3**, 109-121.

Hildt, E., Munz, B., Saher, G., Reifenberg, K., Hofschneider, P. H. (2002). The PreS2 activator MHBs(t) of hepatitis B virus activates c-raf-1/Erk2 signaling in transgenic mice. *EMBO J.*, **21**, 525-535.

Ho, Y. S., Magnenat, J. L., Bronson, R. T., Cao, J., Gargano, M., Sugawara, M., and Funk, C. D. (1997). Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia. *Biol. Chem.*, **272**, 16644-16651.

Homburg, C. H., de Haas, M., von dem Borne, A. E., Verhoeven, A. J., Reutelingsperger, and C. P., Roos, D. (1995). Human neutrophils lose their surface Fc gamma RIII and acquire Annexin V binding sites during apoptosis in vitro. *Blood*, **85**, 532-540.

Hsu, I. C., Metcalf, R. A., Sun, T., Welsh, J. A., Wang, N. J., and Harris, C. C. (1991). Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature*, **350**, 427-428.

Hsu, I. C., Tokiwa, T., Bennett, W., Metcalf, R. A., Welsh, J. A., Sun, T., and Harris, C. C. (1993). p53 gene mutation and integrated hepatitis B viral DNA sequences in human liver cancer cell lines. *Carcinogenesis*, **14**, 987-992.

Hurst, J., Maniar, N., Tombarkiewicz, J., Lucas, F., Roberson, C., Steplewski, Z., James, W., and Perras, J. (1993). A novel model of a metastatic human breast tumour xenograft line. *Br. J. Cancer*, **68**, 274-276.

Imai, H., Narashima, K., Arai, M., Sakamoto, H., Chiba, N., and Nakagawa, Y. (1998). Suppression of leukotriene formation in RBL-2H3 cells that

overexpressed phospholipid hydroperoxide glutathione peroxidase. *J. Biol. Chem.*, **273**, 1990-1997.

İnce, G. (1999). Characterization of Programmed Cell Death Induced By Oxidative Stress in Selenium-deficient Hepatocellular Carcinoma Cells (Master Thesis, Bilkent University).

Itahana, K., Dimri, G., and Campisi, J. (2001). Regulation of cellular senescence by p53. *Eur. J. Biochem.*, **268**, 2784-2791.

Itoh, Y., Iwakiri, S., Kitajima, K., Gotanda T., Miyaki, M., Miyakawa, Y., and Mayumi, M. (1986). Lack of detectable reverse transcriptase activity in human and chimpanzee sera with a high infectivity for non-A, non-B hepatitis. *J. Gen. Virol.*, **67**, 777-779.

Jain, S. K., McVie, R., and Smith, T. (2000). Vitamin E supplementation restores glutathione and malondialdehyde to normal concentrations in erythrocytes of type 1 diabetic children. *Diabetes. Care.*, **23**, 1389-1394.

Jain, S. K., Pemberton, P. W., Smith, A., McMahon, R. F., Burrows, P. C., Aboutwerat, A., and Warnes, T. W. (2002*)*. Oxidative stress in chronic hepatitis C: not just a feature of late stage disease. *J. Hepatol.*, **36**, 805-811.

Jayashree, T., and Subramanyam, C. (2000). Oxidative stress as a prerequisite for aflatoxin production by Aspergillus parasiticus. *Free Radic. Biol. Med.*, **15**, 981-985.

Kadrabova, J., Mad'aric, A., Kovacikova, Z., Podivinsky, F., Ginter, E., and Gazdik, F., (1996). Selenium status is decreased in patients with intrinsic asthma. *Biol. Trace Elem. Res.*, **52**, 241-248.

Kang-Park, S., Lee, J. H., Shin, J. H., Lee, Y. I. (2001). Activation of the IGF-II gene by HBV-X protein requires PKC and p44/p42 map kinase signalings. *Biochem. Biophys. Res. Commun.*, **283**, 303-307.

Kasai, H., Crain, P. F., Kuchino, Y., Nishimura, S., Ootsuyama, A., Tanooka, H. (1986). Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair. *Carcinogenesis*, **7**, 1849-1851.

Kaufmann, S. H., Desnoyers, S., Ottaviano, Y., Davidson, N. E., and Poirier, G. G. (1993). Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res.*, **53**, 3976-3985.

Kinnula, V. L., Everitt, J. I., Mangum, J. B., Chang, L. Y., and Crapo, J. D. (1992). Antioxidant defense mechanisms in cultured pleural mesothelial cells. *Am. J. Respir. Cell. Mol. Biol.*, **7**, 95-103.

Kira, Y., Sato, E. F., and Inoue, M. (2002). Related Association of Cu,Zn-type superoxide dismutase with mitochondria and peroxisomes *Arch. Biochem. Biophys.*, **399**, 96-102.

Kiremidjian-Schumacher, L., Roy, M., Wishe, H. I., Cohen, and M. W., Stotzky, G. (1994). Supplementation with selenium and human immune cell functions. II. Effect on cytotoxic lymphocytes and natural killer cells. *Biol. Trace Elem. Res.*, **41**, 115-127.

Klein, E. A. (2003). Clinical models for testing chemopreventative agents in prostate cancer and overview of SELECT: the Selenium and Vitamin E Cancer Prevention Trial. *Recent Results Cancer Res.*, **163**, 212-225.

Klevay, L. M. (1998). Lack of a recommended dietary allowance for copper may be hazardous to your health. *J Am Coll Nutr.*, **17**, 322-326.

Knekt, P., Aromaa, A., Maatela, J., Alfthan, G., Aaran, R. K. , Hakama, M., Hakulinen, T., Peto, R., and Teppo, L. (1990). Serum selenium and subsequent risk of cancer among Finnish men and women. *J. Natl. Cancer Inst.*, **82**, 864-868.

Knekt, P., Marniemi, J., Teppo, L., Heliovaara, M., and Aromaa, A. (1998). Is low selenium status a risk factor for lung cancer? *Am. J. Epidemiol.*, **148**, 975-982.

Kocaturk, P. A., Siklar, Z., Kavas, G. O., Dallar, Y., and Tanyer, G. (2002). Zinc treatment affects superoxide dismutase activity in growth retardation. *Biol. Trace Elem. Res.*, **90**, 39-46.

Korpela, H. (1990). Hepatic selenium concentration in pigs with microangiopathy (mulberry heart disease)-an animal model for the study of oxidative damage. *Int. J. Vitam. Nutr. Res.*, **60**, 156-158.

Krieger-Brauer, H. I., Kather, H. (1995). Antagonistic effects of different members of the fibroblast and platelet-derived growth factor families on adipose conversion and NADPH-dependent H_2O_2 generation in 3T3 L1-cells. *Biochem. J.*, **307**, 549-556.

Kuwano, H., and Morishita, Y. (1999). Mutation analysis of transforming growth factor beta type II receptor, Smad2, and Smad4 in hepatocellular carcinoma. *Int. J. Oncol.*, **14**, 127-131.

Larrea, E., Beloqui, O., Munoz-Navas, M. A., Civeira, M. P., and Prieto, J. (1998). Superoxide dismutase in patients with chronic hepatitis C virus infection. *Free Radic. Biol. Med.*, **24**, 1235-1241.

Lasfargues, E. Y., Coutinho, W. G., and Redfield, E. S. (1978). Isolation of two human tumor epithelial cell lines from solid breast carcinomas. *Natl. Cancer Inst.*, **61**, 967-978.

Lauerova, L., Kovarik, J., Bartek, J., Rejthar, A., and Vojtesek, B. (1988). Novel monoclonal antibodies defining epitope of human cytokeratin 18 molecule. *Hybridoma*, **7**, 495-504.

Lawrence, R. A., Parkhill, L. K., and Burk, R. F. (1978). Hepatic cytosolic non selenium-dependent glutathione peroxidase activity: its nature and the effect of selenium-deficiency. *Nutr.* **108**, 981-987.

Lee, Y. C., Tang, Y. C., Chen, Y. H., Wong, C. M., and Tsou, A. P. (2003). Selenite-induced survival of HuH7 hepatoma cells involves activation of focal adhesion kinase-phosphatidylinositol 3-kinase-Akt pathway and Rac1. *J. Biol. Chem.*(accepted manuscript).

Lei, D. N., Wang, L. Q., Ruebner, B. H., Hsieh, D. P., Wu, B. F., Zhu, C. R., and Du, M. (1990). Effect of selenium on aflatoxin hepatocarcinogenesis in the rat. *J.Biomed. Environ. Sci.*, **3**, 65-80.

Lelbovitz, A., Wright, W. C., Pathak, S., Siciliano, M. J., and Daniels, W. P. (1979). Detection and analysis of a glucose 6-phosphate dehydrogenase phenotype B cell line contamination. *J. Natl. Cancer Inst.*, **63**, 635-645.

Lewisch, S.A., and Levine. R. L. (1995). Determination of 2-oxohistidine by amino acid analysis. *Anal. Biochem.*, **231**, 440-446.

Li, N. Q., Reddy, P. S., Thyagaraju, K., Reddy, A. P., Hsu, B. L., Scholz, R. W., Tu, C. P., and Reddy, C. C. (1990). Elevation of rat liver mRNA for selenium-dependent glutathione peroxidase by selenium-deficiency. *J. Biol. Chem.*, **265**, 108-113.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, **91**, 479-489.

Liu, L., and Meydani, M. (2002). Combined vitamin C and E supplementation retards early progression of arteriosclerosis in heart transplant patients. *Nutr. Rev.*, **60**, 368-371.

Liu, X., Kim, C. N., Yang, J., Jemmerson , R., and Wang, X. (1996).Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell*, **86**: 147-157.

Loguercio, C., De Girolamo, V., Federico, A., Feng, S. L., Cataldi, V., Del Vecchio-Blanco, C., and Gialanella, G. (1997). Trace elements and chronic liver diseases. *Trace Elem. Med. Biol.*, **11**, 158-161.

Low, S. C., Harney, J. W., and Berry, M. (1995). Cloning and functional characterization of human selenophosphate synthetase, an essential component of selenoprotein synthesis. *J.Biol. Chem.*, **270**, 21659-21664.

Maiorino, M,, Thomas, J. P., Girotti, A. W., and Ursini, F. (1991). Reactivity of phospholipid hydroperoxide glutathione peroxidase with membrane and lipoprotein lipid hydroperoxides. *Free Radic. Res. Commun.*, **12-13**, 131-135.

Mallo, G. V., Rechreche, H., Frigerio, J. M., Rocha, D., Zweibaum, A., Lacasa, M., Jordan, B. R., Dusetti, N. J., Dagorn, J. C., and Iovanna, J. L. (1997). Molecular cloning, sequencing and expression of the mRNA encoding human Cdx1 and Cdx2 homeobox. Down-regulation of Cdx1 and Cdx2 mRNA expression during colorectal carcinogenesis. *Int. J. Cancer*, **74**, 35-44.

Mark, S. D., Qiao, Y. L., Dawsey, S. M., Wu, Y. P., Katki, H., Gunter, E. W., Fraumeni, J. F. Jr., Blot, W. J. , Dong, Z. W., and Taylor, P. R. (2000). Prospective study of serum selenium levels and incident esophageal and gastric cancers. *J. Natl. Cancer Inst.*, **92**, 1753-1763.

Martindale, J. L., and Holbrook, N. J. (2002). Cellular response to oxidative stress: signaling for suicide and survival. *J. Cell. Physiol.*, **192**, 1-15.

Masukawa, T., Nishimura, T., and Iwata, H. (1984). Differential changes of glutathione S-transferase activity by dietary selenium. *Biochem. Pharmacol.*, **33**, 2635-2639.

Mates M. (2000). Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. *Toxicology*, **153**, 83-104.

Mates, J. M., and Sanchez-Jimenez, F. (1999). Antioxidant enzymes and their implications in pathophysiologic processes. *Front. Biosci.*, **4**, 339-345.

McCord, J. M., and Fridovich, I. (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.*, **244**, 6049-6055.

McKeehan, W. L., Hamilton, W. G., and Ham, R. G. (1976). Selenium is an essential trace nutrient for growth of WI-38 diploid human fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.*, **73**, 2023-2027.

McLeod, R., Ellis, E. M., Arthur, J. R., Neal, G. E., Judah, D. J., Manson, M. M., and Hayes, J. D. (1997). Protection conferred by selenium-deficiency against

aflatoxin B1 in the rat is associated with the hepatic expression of an aldo-keto reductase and a glutathione S-transferase subunit that metabolize the mycotoxin. *Cancer Res.*, **57**, 4257-4266.

Meier, B., Cross, A. R., Hancock, J. T., Kaup, F. J., and Jones, O. T. (1991). Identification of a superoxide-generating NADPH oxidase system in human fibroblasts. *Biochem. J.*, **275**, 241-245.

Meier, B., Radeke, H. H., Selle, S., Younes, M., Sies, H., Resch, K., Habermehl, G. G. (1989). Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumour necrosis factor-alpha. *Biochem. J.*, **263**, 539-545.

Meydani, S. N., Meydani, M., Blumberg, J. B., Leka, L. S., Siber, G., Loszewski, R., Thompson, C., Pedrosa, M. C., Diamond, R. D., and Stollar, B. D. (1997). Vitamin E supplementation and in vivo immune response in healthy elderly subjects. A randomized controlled trial. *JAMA.*, **277**, 1380-1386.

Meydani, S. N., Meydani, M., Blumberg, J. B., Leka, L. S., Siber, G., Loszewski, R., and Thompson, C. (1998). Vitamin E supplementation and in vivo immune response in healthy elderly subjects. A randomized controlled trial. *JAMA.*, **279**, 505; discussion 505-506.

Miller, D. K., Sadowski, S., Soderman, D. D., and Kuehl, F. A. Jr. (1985). Endothelial cell prostacyclin production induced by activated neutrophils. *J. Biol. Chem.*, **260**, 1006-1014.

Mirault, M. E.,, Tremblay, A., Beaudoin, N., and Tremblay, M. (1991). Overexpression of seleno-glutathione peroxidase by gene transfer enhances the resistance of T47D human breast cells to clastogenic oxidants. *J. Biol. Chem.*, **266**, 20752-20760.

Moir, D. C., and Masters, H. G. (1979). Hepatosis dietetica, nutritional myopathy, mulberry heart disease and associated hepatic selenium level in pigs. *Aust. Vet. J.*, **55**, 360-364.

Morales, A., Garcia-Ruiz, C., Miranda, M., Mari, M., Colell, A., Ardite, E., and Fernandez-Checa, J. C. (1997). Tumor necrosis factor increases hepatocellular

glutathione by transcriptional regulation of the heavy subunit chain of gamma-glutamylcysteine synthetase. *J. Biol. Chem.*, **272**, 30371-30379.

Mori, A., Hiramatsu, M., Yokoi, I., and Edamatsu, R. (1990). Biochemical pathogenesis of post-traumatic epilepsy. *Pavlov J. Biol. Sci.*, **25**, 54-62.

Morikawa, K., Walker, S. M., Nakajima, M., Pathak, S., Jessup, J. M., and Fidler, I. (1988). Influence of organ environment on the growth, selection, and metastasis of human colon carcinoma cells in nude mice. *J. Cancer Res.*, **48**, 6863-6871.

Moriya, K., Fujie, H, Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, K., Matsuura, Y., Kimura, S., Miyamura, T., and Koike, K. (1998). The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat. Med.*, **4**, 1065-1067.

Mulder, T. P., Janssens, A. R., de Bruin, W. C., Peters, W. H., Cooreman, M. P., and Jansen, J. B. (1997). Plasma glutathione S-transferase alpha 1-1 levels in patients with chronic liver disorders. *Clin. Chim. Acta.*, **258**, 69-77.

Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T., and Sato, J. (1982). Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. *Cancer Res.*, **42**, 3858-3863.

Nhieu, J. T., Renard, C. A., Wei, Y., Cherqui, D., Zafrani, E. S., and Buendia, M. A. (1999). Nuclear accumulation of mutated beta-catenin in hepatocellular carcinoma is associated with increased cell proliferation. *Am. J. Pathol.*, **155**, 703-710.

Niederau, C., Fischer, R., Sonnenberg, A., Stremmel, W., Trampisch, H. J., and Strohmeyer, G. (1985). Survival and causes of death in cirrhotic and in noncirrhotic patients with primary hemochromatosis. *N. Engl. J. Med.*, **313**, 1256-1262.

Niu, J., Kumar, U., Monjardino, J., Goldin, R., Rosin, D., Thomas, H. C. (1995). Hepatitis C virus replication in hepatocellular carcinoma. *J. Clin. Pathol.*, **48**, 880-882.

Nomura, K., Imai, H., Koumura, T., Kobayashi, T., and Nakagawa, Y. (2000). Mitochondrial phospholipid hydroperoxide glutathione peroxidase inhibits the release of cytochrome c from mitochondria by suppressing the peroxidation of cardiolipin in hypoglycaemia-induced apoptosis. *Biochem. J.*, **351**, 183-193.

Nordberg, J., and Arner, E. S. (2001). Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic. Biol. Med.*, **31**, 1287-1312.

Ohnishi, K., Iida, S., Iwama, S., Goto, N., Nomura, F., Takashi, M., Mishima, A., Kono, K., Kimura, K., Musha, H., Kotota, K., Okuda, K. (1982). The effect of chronic habitual alcohol intake on the development of liver cirrhosis and hepatocellular carcinoma: relation to hepatitis B surface antigen carriage. *Cancer*, **49**, 672-677.

Okuda, M., Li, K., Beard, M. R., Showalter, L. A., Scholle, F., Lemon, S. M., and Weinman, S. A. (2002). Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology*, **122**, 366-375.

Ostrakhovitch, E. A., Lordnejad, M. R., Schliess, F., Sies, H., and Klotz, L. O. (2002). . Copper ions strongly activate the phosphoinositide-3-kinase/Akt pathway independent of the generation of reactive oxygen species. *Arch. Biochem. Biophys.*, **397**, 232-239,

Öztürk, M. (1994). Chromosomal Rearrangements and Tumor Suppressor Genes in Primary Liver Cancer. **Ch18**, 269-277.

Öztürk, M. (1999). Genetic aspects of hepatocellular carcinogenesis. *Semin. Liver Dis.*, **19**, 235-242,

Öztürk, M., and Çetin-Atalay, R. (2002). Biology of Hepatocellular Carcinoma. Section **7**, **Ch43,** 1-16**.**

Packer, L., Weber, S. U., and Rimbach, G. (2001). Molecular aspects of alpha-tocotrienol antioxidant action and cell signalling. *Nutr.*, **131**, 369S-373S.

Palomba L., Sestili, P., Columbaro, M., Falcieri, E., and Cantoni, O. (1999). Apoptosis and necrosis following exposure of U937 cells to increasing concentrations of hydrogen peroxide: the effect of the poly(ADP-ribose)polymerase inhibitor 3-aminobenzamide. *Biochem. Pharmacol.*, **58**, 1743-1750.

Park, H. S., Park, E., Kim, M. S., Ahn, K., Kim, I. Y., and Choi, E. J. (2000a). Selenite inhibits the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) through a thiol redox mechanism. *J. Bio.l Chem.*, **275**, 2527-2531.

Park, H. S., Huh, S. H., Kim, Y., Shim, J., Lee, S. H., Park, I. S., Jung, Y. K., Kim, I. Y., and Choi, E. J. (2000b). Selenite negatively regulates caspase-3 through a redox mechanism. *J Biol Chem*, **275**, 8487-8491.

Park, J. G., Lee, J. H., Kang, M. S., Park, K. J., Jeon, Y. M., Lee, H. J., Kwon, H. S., Park, H. S., Yeo, K. S., Lee, K. U., et al. (1995). Characterization of cell lines established from human hepatocellular carcinoma. *Int. J. Cancer*, **62**, 276-282.

Parkin, D. M., and Muir, C. S. (1992). Cancer Incidence in Five Continents. Comparability and quality of data.. *IARC Sci. Publ.*, **120**, 45-173.

Parkin, D. M., Bray, F. I., and Devesa, S. S. (2003). Corrigendum to "Cancer burden in the year 2000. The global picture" [European Journal of Cancer, 37(Suppl. 8) (2001) S4-S66]. *Eur. J. Cancer*, **39**, 848.

Parkin, D. M., Pisani, P., and Ferlay, J. (1999). Estimates of the worldwide incidence of 25 major cancers in 1990. *Int. J. Cancer.*, **80**, 827-841.

Percival, S. S. (1995). Neutropenia caused by copper deficiency: possible mechanisms of action. *Nutr. Rev.*, **53**, 59-66.

Perez, M. J., and Cederbaum, A. I. (2003). Proteasome inhibition potentiates CYP2E1-mediated toxicity in HepG2 cells. *Hepatology*, **37**, 1395-1404.

Pineau, P., Nagai, H., Prigent, S., Wei, Y., Gyapay, G., Weissenbach, J., Tiollais, P., Buendia, M. A., and Dejean, A. (1999). Identification of three distinct regions

of allelic deletions on the *short* arm of chromosome 8 in hepatocellular carcinoma. *Oncogene*, **18**, 3127-3134.

Plachta, H., Bartnikowska, E., and Obara, A. (1992). Lipid peroxides in blood from patients with atherosclerosis of coronary and peripheral arteries. *Clin. Chim. Acta.*, **211**, 101-112.

Pocard, M., Bras-Goncalves, R., Hamelin, R., Northover, J., and Poupon, M. F. (2000). Response to 5-fluorouracil of orthotopically xenografted human colon cancers with a microsatellite instability: influence of P53 status. *Anticancer Res.*, **20**, 85-90.

Prasad, A. S., Fitzgerald, J. T., Hess, J. W., Kaplan, J., Pelen, F., and Dardenne, M. (1993). Zinc deficiency in elderly patients. *Nutrition.*, **9**, 218-224.

Prinsze, C., Dubbelman, T. M., and Van Steveninck, J. (1990). Protein damage, induced by small amounts of photodynamically generated singlet oxygen or hydroxyl radicals. *Biochim. Biophys. Acta*, **1038**, 152-157.

Qadri, I., Iwahashi, M., and Simon, F. (2002). Hepatitis C virus NS5A protein binds TBP and p53, inhibiting their DNA binding and p53 interactions with TBP and ERCC3. *Biochim. Biophys. Acta*, **1592**, 193-204.

Qin, L. X., Tang, Z. Y., Sham, J. S., Ma, Z. C., Ye, S. L., Zhou, X. D., Wu, Z. Q., Trent, J. M., Guan, X. Y. (1999). The association of chromosome 8p deletion and tumor metastasis in human hepatocellular carcinoma. *Cancer Res.*, **59**, 5662-5665.

Qin, S., and Chock, P. B. (2003). Implication of phosphatidylinositol 3-kinase membrane recruitment in hydrogen peroxide-induced activation of PI3K and Akt.
Quinlan, G. J., Evans, T. W., Gutteridge, and J. M. (2002). Iron and the redox status of the lungs. *Free Radic. Biol. Med.*, **33**, 1306-1313.

Rafferty, T. S., Beckett, G. J., Walker, C., Bisset, Y. C., and McKenzie, R. C. (2003). Selenium protects primary human keratinocytes from apoptosis induced by exposure to ultraviolet radiation. *Clin. Exp. Dermatol.*, **28**, 294-300.

Rakowicz-Szulczynska, E. M., Steplewski, Z., and Koprowski, H. (1992). Nuclear translocation of monoclonal antibody directed against cell-surface carbohydrate Y determinant. *Am. J. Pathol.*, **141**, 937-947.

Rayman, M. P. (2000).The importance of selenium to human health. *Lancet*, **356**, 233-241.

Reaume, A. G., Elliott, J. L., Hoffman, E. K., Kowall, N. W., Ferrante, R. J., Siwek, D. F., Wilcox, H. M., Flood, D. G., Beal, M. F., Brown, R. H. Jr., Scott, R. W., and Snider, W. D. (1996). Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat. Genet.*, **13**, 43-47.

Reisbach, G., Gebhart, E., and Cailleau, R. (1982). Sister chromatid exchanges and proliferation kinetics of human metastatic breast tumor cells lines. *Anticancer Res.*, **2**, 257-260.

Reyes, H. (1997). Intrahepatic cholestasis. A puzzling disorder of pregnancy. *J. Gastroenterol. Hepatol.*, 12: 211-216.

Ricciarelli, R., Zingg, J. M., and Azzi, A. (2001). Vitamin E: protective role of a Janus molecule. *FASEB J.*, **15**, 2314-2325.

Richter, C., Gogvadze, V., Laffranchi, R., Schlapbach, R., Schweizer, M., Suter, M., Walter-Royall, J. A., and Ischiropoulos, H. (1993). Evaluation of 2',7'-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. *Arch. Biochem. Biophys.*, **302**, 348-355.

Rigamonti, C., Mottaran, E., Reale, E., Rolla, R., Cipriani, V., Capelli, F., Boldorini, R., Vidali, M., Sartori, M., and Albano,E. (2003). Moderate alcohol consumption increases oxidative stress in patients with chronic hepatitis C *Hepatology*, **38**, 42-49.

Rigo, A., Stevanato, R., Finazzi-Agro, A., and Rotilio, G. (1977). An attempt to evaluate the rate of the Haber-Weiss reaction by using OH radical scavengers. *FEBS Lett.*, **80**, 130-132.

Rizk, N. N. (1982). Scanning electron microscopic study of three human breast cancer cell lines. *Acta Cytol.*, **26**, 714-720.

Robinson, W. S., Klote, L., and Aoki N. (1990). Hepadnaviruses in cirrhotic liver and hepatocellular carcinoma. *J. Med. Virol.*, **31**, 18-32.

Rogler, C. E., Hino, O., and Su, C. Y. (1987). Molecular aspects of persistent woodchuck hepatitis virus and hepatitis B virus infection and hepatocellular carcinoma. *Hepatology*, **7**, 74S-78S.

Rouach, H., Fataccioli, V., Gentil, M., French, S. W., Morimoto, M., and Nordmann, R. (1997). Effect of chronic ethanol feeding on lipid peroxidation and protein oxidation in relation to liver pathology. *Hepatology*, **25**, 351-355.

Saari, J. T., and Schuschke, D. A. (1999). Cardiovascular effects of dietary copper deficiency. *Biofactors*, **10**, 359-75.

Salgueiro, M. J., Zubillaga, M. B., Lysionek, A. E., Caro, R. A., Weill, R., and Boccio, J. R. (2002). The role of zinc in the growth and development of children. *Nutrition*, **18**, 510-519.

Sappey, C., Legrand-Poels, S., Best-Belpomme, M., Favier, A., Rentier, B., and Piette, J. (1994). Stimulation of glutathione peroxidase activity decreases HIV type 1 activation after oxidative stress. *AIDS Res. Hum. Retroviruses*, **10**, 1451-1461.

Sato, Y., Hotta, N., Sakamoto, N., Matsuoka, S., Ohishi, N., and Yagi, K. (1979). Lipid peroxide level in plasma of diabetic patients. *Biochem. Med.*, **21**, 104-107.

Satoh, S., Daigo, Y., Furukawa, Y., Kato, T., Miwa, N., Nishiwaki, T., Kawasoe, T., Ishiguro, H., Fujita, M., Tokino, T., Sasaki, Y., Imaoka, S., Murata, M., Shimano, T., Yamaok,a Y., Nakamura, Y. (2000). AXIN1 mutations in

hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. *Nat. Genet.*, **24**, 245-250.

Sayan, A. E., Sayan, B. S., Findikli, N.,and Ozturk, M. (2001a). Acquired expression of transcriptionally active p73 in hepatocellular carcinoma cells. *Oncogene*, **20**, 5111-5117.

Sayan, B. S., Ince, G., Sayan, A. E., and Ozturk, M. (2001b). NAPO as a novel marker for apoptosis. *J. Cell. Biol.*, **155**, 719-724.

Schuckelt, R., Brigelius-Flohe, R., Maiorino, M., Roveri, A., Reumkens, J., Strassburger, W., Ursini, F., Wolf, B., and Flohe, L. (1991). Phospholipid hydroperoxide glutathione peroxidase is a selenoenzyme distinct from the classical glutathione peroxidase as evident from cDNA and amino acid sequencing. *Free Radic. Res. Commun.*, **14**, 343-361.

Schultz, R. M., Woods, W. A., and Chirigos, M. A. (1975). Detection in colorectal carcinoma patients of antibody cytotoxic to established cell strains derived from carcinoma of the human colon and rectum. *Int. J. Cancer*, **16**, 16-23.

Schwarz, K., and Foltz, C. M. (1999). Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. *Nutrition*, **15**: 255.

Sells, M. A., Chen, M. L., and Acs, G. (1987). Production of hepatitis B virus particles in HepG2 cells transfected with cloned hepatitis B virus DNA. *Proc. Natl. Acad. Sci. U.S.A.,* **84**: 1005-1009.

Sentman, M. L., Jonsson, L. M., and Marklund, S. L. (1999). Enhanced alloxan-induced beta-cell damage and delayed recovery from hyperglycemia in mice lacking extracellular-superoxide dismutase. *Free Radic. Biol. Med.*, **27**, 790-796.

Shen, H. M., Ong, C. N., and Shi, C. Y. (1995). Involvement of reactive oxygen species in aflatoxin B1-induced cell injury in cultured rat hepatocytes. *Toxicology*, **99**, 115-123.

Shen, H. M., Shi, C. Y., Shen, Y., and Ong, C. N. (1996). Detection of elevated reactive oxygen species level in cultured rat hepatocytes treated with aflatoxin B1. *Free Radic. Biol. Med.*, **21**, 139-146.

Shen, H. M., Yang, C. F., and Ong, C. N. (1999). Sodium selenite-induced oxidative stress and apoptosis in human hepatoma HepG2 cells. *Int. J. Cancer.*, **81**, 820-828.

Shidoji, Y., Hayashi, K., Komura, S., Ohishi, N., and Yagi, K. (1999). Loss of molecular interaction between cytochrome c and cardiolipin due to lipid peroxidation. *Biochem. Biophys. Res. Commun.*, **264**, 343-347.

Shimotohno, K., Watash,i K., Tsuchihara, K., Fukuda, K., Marusawa, H., and Hijikata, M. (2002). Hepatitis C virus and its roles in cell proliferation. *J. Gastroentero.*, **37 Suppl 13**, 50-54.

Simon, D., and Carr, B. I. (1995). Integration of hepatitis B virus and alteration of the 1p36 region found in cancerous tissue of primary hepatocellular carcinoma with viral replication evidenced only in noncancerous, cirrhotic tissue. *Hepatology*, **22**, 1393-1398.

Sipowicz, M. A., Chomarat, P., Diwan, B. A., Anver, M. A., Awasthi, Y. C., Ward, J. M., Rice, J.M., Kasprzak, K. S., Wild, C. P., and Anderson, L. M. (1997). Increased oxidative DNA damage and hepatocyte overexpression of specific cytochrome P450 isoforms in hepatitis of mice infected with Helicobacter hepaticus. *Am. J. Pathol.*, **151**, 933-941.

Stadtman, E. R. (1992). Protein oxidation and aging. *Science*, **257**, 1220-1224.

Stahl, W., and Sies, H. (2002). http://www.uni-dusseldorf.de/.

Stapleton, S. R., Garlock, G. L., Foellmi-Adams, L., and Kletzien, R. F. (1997). Selenium: potent stimulator of tyrosyl phosphorylation and activator of MAP kinase. *Biochim. Biophys. Acta*, **1355,** 259-269.

Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J C., and Witztum, J. L. (1989). Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.*, **320**, 915-924.

Stiles, N. J., and Boosalis, M. G. (1995). Case report of zinc deficiency in an elderly woman. *Clin. Lab. Sci.*, **8**, 39-42.

Stridh, H., Kimland, M., Jones, D. P., Orrenius, S., and Hampton, M. B. (1998). Cytochrome c release and caspase activation in hydrogen peroxide- and tributyltin-induced apoptosis. *FEBS Lett.*, **429**, 351-355.

Sun, Y., Elwell, J. H., and Oberley, L. W. (1988). A simultaneous visualization of the antioxidant enzymes glutathione peroxidase and catalase on polyacrylamide gels. *Free Radic. Res. Commun.*, **5**, 67-75.

Sundaresan, M., Yu, Z. X., Ferrans, V. J., Irani, K., Finkel, T. (1995). Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science*, **270**, 296-299.

Syburra, C., and Passi, S. *Ukr.* (1999). Oxidative stress in patients with multiple sclerosis. *Biokhim. Zh.*, **71**, 112-115.

Tabor, E. (2001). *Dig. Liver Dis.*, **33**, 115-117.

Tai, D. I., Tsai, S. L., Chen, Y. M., Chuang, Y. L., Peng, C. Y., Sheen, I. S., Yeh, C. T., Chang, K. S., Huang, S N., Kuo, G.C., and Liaw, Y. F. (2000). Activation of nuclear factor kappaB in hepatitis C virus infection: implications for pathogenesis and hepatocarcinogenesis. *Hepatology*, **31**, 656-664.

Takahashi, K., Avissar, N., Whitin, J., and Cohen, H. (1987). Purification and characterization of human plasma glutathione peroxidase: a selenoglycoprotein distinct from the known cellular enzyme. *Arch. Biochem. Biophys.*, **256**, 677-686.

Takahashi, K., Newburger, P. E., and Cohen, H. J. (1986). Glutathione peroxidase protein. Absence in selenium-deficiency states and correlation with enzymatic activity. *J. Clin. Invest.*, **77**, 1402-1404.

Takeda, S., Shibata, M., Morishima, T., Harada, A., Nakao, A., Takagi, H., Nagai, Y. (1992). Hepatitis C virus infection in hepatocellular carcinoma. Detection of plus-strand and minus-strand viral RNA. *Cance*r, **70**, 2255-2259.

Tannapfel, A., and Wittekind, C. (2002). Genes involved in hepatocellular carcinoma: deregulation in cell cycling and apoptosis.*Virchows Arch.*, **440**, 345-352.

Tannapfel, A., Wasner, M., Krause, K., Geissler, F., Katalinic, A., Hauss, J., Mossner, J., Engeland, K., and Wittekind, C. (1999). Expression of p73 and its relation to histopathology and prognosis in hepatocellular carcinoma. *J. Natl. Cancer Inst.*, **91**, 1154-1158.

Tapiero, H., Gaté, L., and Tew, K. D. (2001). Iron: deficiencies and requirements. *Biomed. Pharmacother.*, **55**, 324-332.

Thannickal, V. J., and Fanburg, B. L. (1995). Activation of an H_2O_2-generating NADH oxidase in human lung fibroblasts by transforming growth factor beta 1. *J. Biol. Chem.*, **270**, 30334-30338.

Thannickal, V. J., and Fanburg, B. L. (2000). Reactive oxygen species in cell signaling. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **279**, 1005-1028.

Thannickal, V. J., Day, R. M., Klinz, S. G., Bastien, M. C., Larios, J. .M, and Fanburg, B. L. (2000). Ras-dependent and -independent regulation of reactive oxygen species by mitogenic growth factors and TGF-beta1. *FASEB J.*, **14**, 1741-1748.

Thorgeirsson, S. S., and Grisham, J. W. (2002) Molecular pathogenesis of human hepatocellular carcinoma. *Nat. Genet.*, **31**, 339-346

Tinggi, U. (2003). Essentiality and toxicity of selenium and its status in Australia. *Toxicol. Lett.*, **137**, 103-110.

Toyoda, H., Himeno, S., and Imura, N. (1989). The regulation of glutathione peroxidase gene expression relevant to species difference and the effects of dietary selenium manipulation. *Biochim. Biophys. Acta,* **1008**, 301-308.

Toyokuni, S., Okamoto, K., Yodoi, J., and Hiai, H. (1995). Persistent oxidative stress in cancer. *FEBS Lett.*, **358**, 1-3.

Tsang, K. Y., Fudenberg, H. H., and Gnagy, M. J. (1981). Osteosarcoma patients: isolation of serum antibodies by affinity chromatography. *J. Natl. Cancer. Inst.*, **67**, 1183-1189.

Ünsal, H., Yakicier, C., Marcais, C., Kew, M., Volkmann, M., Zentgraf, H., Isselbacher, K. J., and Ozturk, M. (1994). Genetic heterogeneity of hepatocellular carcinoma. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 822-826.

Valgimigli, M., Valgimigli, L., Trere, D., Gaiani, S., Pedulli, G. F., Gramantieri, L., and Bolondi, L. (2002). Oxidative stress EPR measurement in human liver by radical-probe technique. Correlation with etiology, histology and cell proliferation. *Free Radic. Res.*, **36**, 939-948.

Valimaki M. J., Harju, K. J., Ylikahri, R. H. (1983). Decreased serum selenium in alcoholics--a consequence of liver dysfunction. *Clin. Chim. Acta*, **130**, 291-296.

Verhoven, B., Schlegel, R. A., and Williamson, P. (1995). Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. *J. Exp. Med.*, **182**, 1597-1601.

Vermes, I., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods*, **184**, 39-51.

Vernet, P., Rock, E., Mazur, A., Rayssiguier, Y., Dufaure, J. P., and Drevet, J. R. (1999). Selenium-independent epididymis-restricted glutathione peroxidase 5 protein (GPX5) can back up failing Se-dependent GPXs in mice subjected to selenium-deficiency. *Mol. Reprod. Dev.*, **54**, 362-370.

Vic, P., Vignon, F., Derocq, D., and Rochefort, H. (1982). Effect of estradiol on the ultrastructure of the MCF7 human breast cancer cells in culture. *Cancer Res.*, **42**, 667-673.

von Herbay, A., Stahl, W., Niederau, C., and Sies, H. (1997). Vitamin E improves the aminotransferase status of patients suffering from viral hepatitis C: a randomized, double-blind, placebo-controlled study. *Free Radic. Res.*, **27**, 599-605.

von Herbay, A., Stahl, W., Niederau, C., von Laar, J., Strohmeyer, G., and Sies, H. (1996). Diminished plasma levels of vitamin E in patients with severe viral hepatitis. *Free Radic. Res.*, **25**, 461-466.

von Kleist, S., Chany, E., Burtin, P., King, M., and Fogh, J. (1975). Immunohistology of the antigenic pattern of a continuous cell line from a human colon tumor. *J. Natl. Cancer Inst.*, **55**, 555-650.

Wallach, J. D., Lan, M., Yu, W. H., Gu, B. Q., Yu, F. T., and Goddard, R. F. (1990). Common denominators in the etiology and pathology of visceral lesions of cystic fibrosis and Keshan disease. *Biol. Trace Elem. Res.*, **24**, 189-205.

Wang, J., Zindy, F., Chenivesse, X., Lamas, E., Henglein, B., and Brechot, C. (1992). Modification of cyclin A expression by hepatitis B virus DNA integration in a hepatocellular carcinoma. *Oncogene*, **7**, 1653-1656.

Wang, X. W., Hussain, S. P., Huo, T. I., Wu, C. G., Forgues, M., Hofseth, L.J., Brechot, C., and Harris, C.C. (2002). Molecular pathogenesis of human hepatocellular carcinoma. *Toxicology*, **181-182**, 43-47.

Wang, X., McCullough, K. D., Franke, T. F., and Holbrook, N. J. (2000). Epidermal growth factor receptor-dependent Akt activation by oxidative stress enhances cell survival. *J. Biol Chem.*, **275**, 14624-14631.

Waris, G., Huh, K. W., and Siddiqui, A. (2001). Mitochondrially associated hepatitis B virus X protein constitutively activates transcription factors STAT-3 and NF-kappa B via oxidative stress. *Mol. Cell. Biol.*, **21**, 7721-7730.

Weisiger, R. A., and Fridovich, I. (1973). Mitochondrial superoxide simutase. Site of synthesis and intramitochondrial localization. *J. Biol. Chem.*, **248**, 4793-4796.

Wells, P. G., and Winn, L. M. (1996). Biochemical toxicology of chemical teratogenesis. *Crit. Rev. Biochem. Mol. Biol.*, **31**, 1-40.

Willett, W. C., Polk, B. F., Morris, J. S., Stampfer, M. J., Pressel, S., Rosner, B., Taylor, J. O. , Schneide, K., and Hames, C. G. (1983). Prediagnostic serum selenium and risk of cancer. *Lancet*, **2**, 130-134.

Win, N., Okada, S., Win, K. M., Taketa, K., Akiyama, T., Aye, K. S., and Kying, H. (2000). Hemoglobinopathies, Iron Overload and Chronic Viral Hepatitis in Patients with Hepatocellular Carcinoma in Myanmar. *Asian Pac. J. Cancer Prev.*, **1**, 127-130.

Wong, C. M., Fan, S. T., and Ng, I. O. (2001). beta-Catenin mutation and overexpression in hepatocellular carcinoma: clinicopathologic and prognostic significance. *Cancer*, **92**, 136-145.

Yakicier, M. C., Irmak, M. B., Romano, A., Kew, M., and Ozturk, M. (1999). Smad2 and Smad4 gene mutations in hepatocellular carcinoma. *Oncogene*, **18**, 4879-4883.

Yakicier, M. C., Legoix, P., Vaury, C., Gressin, L., Tubacher, E., Capron, F., Bayer, J., Degott, C., Balabaud, C., and Zucman-Rossi, J. (2001). Identification of homozygous deletions at chromosome 16q23 in aflatoxin B1 exposed hepatocellular carcinoma. *Oncogene*, **20**, 5232-5238.

Yan, H., and Harding, J. J. (1997). Glycation-induced inactivation and loss of antigenicity of catalase and superoxide dismutase. *Biochem. J.*, **328**, 599–605.

Yang, Y., Sharma, R., Zimniak, P., and Awasthi, Y. C. (2002). Role of alpha class glutathione S-transferases as antioxidant enzymes in rodent tissues. *Toxicol. Appl. Pharmacol.*,**182**, 105-115.

Yang, C. F., Liu, J., Wasser, S., Shen, H. M., Tan, C. E., and Ong, C. N. (2000). Inhibition of ebselen on aflatoxin B(1)-induced hepatocarcinogenesis in Fischer 344 rats. *Carcinogenesis*, **21**, 2237-2243.

Yao, Y. J., Ping, X. L., Zhang, H., Chen, F. F., Lee, P. K., Ahsan, H., Chen, C. J., Lee, P. H., Peacocke, M., Santella, R. M., and Tsou, H. C. (1999). PTEN/MMAC1 mutations in hepatocellular carcinomas. *Oncogene*, **18**, 3181-3185.

Yoo, Y. D., Ueda, H., Park, K., Flanders, K. C., Lee, Y. I., Jay, G., and Kim, S. J. (1996). Regulation of transforming growth factor-beta 1 expression by the hepatitis B virus (HBV) X transactivator. Role in HBV pathogenesis. *J. Clin. Invest.*, **97**, 388-395

Yoshimura, S., Watanabe, K., Suemizu, H., Onozawa, T., Mizoguchi, J., Tsuda, K., Hatta, H., and Moriuchi, T. (1991). Tissue specific expression of the plasma glutathione peroxidase gene in rat kidney. *J. Biochem., (Tokyo)*, **109**, 918-923.

Yoshizawa, K., Willett, W. C., Morris, S. J., Stampfer, M. J., Spiegelman, D., Rimm, E. B., Giovannucci, E. (1998). Study of prediagnostic selenium level in toenails and the risk of advanced prostate cancer. *J. Nat.l Cancer Inst.*, **90**, 1219-1224.

Yost, F. J. Jr., and Fridovich, I. (1976). Superoxide and hydrogen peroxide in oxygen damage. *Arch. Biochem. Biophys.*, **175**, 514-519.

Youn, H. D., Kim, E. J., Roe, J. H., Hah, Y. C., and Kang, S. O. (1996). A novel nickel-containing superoxide dismutase from Streptomyces spp. *Biochem. J.*, **318**, 889-896.

Yu, M. W., Horng, I. S., Hsu, K. H., Chiang, Y. C., Liaw, Y. F., and Chen, C. (1999). Plasma selenium levels and risk of hepatocellular carcinoma among men with chronic hepatitis virus infection. J.*Am. J. Epidemiol.*, **150**, 367-374.

Yu, S. Y., Zhu, Y. J., and Li, W. G. (1997). Protective role of selenium against hepatitis B virus and primary liver cancer in Qidong. *Biol. Trace Elem. Res.*, **56**: 117-124.

Zelko, I. N., Mariani, T. J., and Folz, R. J. (2002). Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radic. Biol. Med.*, **33**, 337-349.

Zhao, X., Li, J., He, Y., Lan, F., Fu, L., Guo, J., Zhao, R., Ye, Y., He, M., Chong, W., Chen, J., Zhang, L., Yang, N., Xu, B., Wu, M., Wan, D. and , Gu, J. (2001). A novel growth suppressor gene on chromosome 17p13.3 with a high frequency of mutation in human hepatocellular carcinoma. *Cancer Res.*, **61**, 7383-7387.

Zhuang, S., Ouedraogo, G. D., and Kochevar, I. E. (2003). Downregulation of epidermal growth factor receptor signaling by singlet oxygen through activation of caspase-3 and protein phosphatases. *Oncogene*, **22**, 4413-4424.

Zigler, J. S. Jr., Jernigan, H. M. Jr., Garland, D., and Reddy, V. N. (1985). The effects of "oxygen radicals" generated in the medium on lenses in organ culture: inhibition of damage by chelated iron. *Arch. Biochem. Biophys.*, **241**, 163-172.

Zimniak, L., Awasthi, S., Srivastava, S. K., and Zimniak, P. (1997). Increased resistance to oxidative stress in transfected cultured cells overexpressing glutathione S-transferase mGSTA4-4. *Toxicol. Appl. Pharmacol.*, **143**, 221-229.