N,N,N',N'-Tetrakis (2-pyridylmethyl) Ethylenediamine-Induced Depletion of

the Labile Intracellular Pool of Zinc Suppressed the Growth of

Human Breast Cancer Cells

by

Bella Wen Wu

B.Sc.(Biochemistry), The University of British Columbia, 2000

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Human Nutrition Program)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 2003

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of ___HUMAN NUTRITION___

The University of British Columbia
Vancouver, Canada

Date ___April 23/03___

ABSTRACT

Zinc is critically involved in many physiological processes including growth. Increasing evidence has revealed the importance of the labile intracellular pool of zinc (LIPZ) in zinc-mediated growth regulation, especially apoptosis, or programmed cell death. However, the mechanisms whereby zinc regulates apoptosis are largely unknown. Zinc has been shown to be associated with several key apoptotic regulatory factors i.e. p53 and members of the Bcl-2 family. We hypothesized that deprivation of LIPZ would reduce cell survival and promote cell death in human breast cancer cells. The overall objectives were to investigate the effects of LIPZ depletion on the growth of human breast cancer cells and to explore the possible mechanisms involved. Three human breast cancer cell lines (MCF-7, MDA-MB-231 and T47D) and a fibrocystic breast cell line (MCF-10), were treated with various concentrations of the intracellular zinc chelator (NNN'N'-tetrakis(2-pyridylmethyl) ethylenediamine; TPEN). After various incubation durations, total cellular zinc concentration and the LIPZ size were quantitated. Cell growth was assessed using cell number counts, cell viability and profiles of cell survival and cell death (necrosis and apoptosis). The expression profile of p53, gadd45, bcl-2 and bax were determined using reverse transcription–polymerase chain reaction (RT-PCR). TPEN induced a dose-dependent reduction in the LIPZ size, but had no effect on the total cellular zinc concentration. TPEN-induced depletion of LIPZ led to decreased cell viability and survival, and increased necrosis and apoptosis, indicating that an adequate size of LIPZ was critical to the growth of breast cancer and fibrocystic breast cells. The depletion of LIPZ was also associated with alterations in p53, gadd45, Bcl-2 and Bax mRNA levels in two of the breast cancer cell lines (MDA-MB-231 and T47D) investigated. Overall, this study demonstrated that LIPZ was important

to breast cancer cell growth. The TPEN-induced depletion LIPZ was cell line-specific, and was independent of total cellular zinc content. The reduced LIPZ size was associated with reduced growth of human breast cancer cells through increased cell death in a cell line-specific manner. The elevated levels of apoptotic cell death were associated with altered mRNA level of apoptotic regulatory genes.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

CHAPTER I.

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer among Canadian women, with an incidence rate of 20,500 new cases per year (Canadian Cancer Statistics, 2002). Despite a steady decline in mortality rates due to advances in cancer treatment, breast cancer continues to be one of the leading causes of death in women with cancer in Canada, with approximately 5,400 deaths reported in 2002.

Tumourigenesis is characterized by uncontrolled and excessive cell proliferation, and the dysregulation of normal apoptosis has been postulated as the cause of this imbalance in cell growth (Krajewski et al., 1999). Growth can be viewed as the net balance between two complementing processes: cell division (mitosis) and cell death (apoptosis or programmed cell death and necrosis). The maintenance of this homeostatic balance is essential for the physiological well being of organisms, such as humans. The failure to sustain the balance may well underlie the development of certain diseases (Saikumar et al., 1999). Mutation, overexpression and decreased expression of genes and proteins involved in apoptosis have been implicated in breast cancer pathogenesis and progression (Carolin and Pass, 2000). Hence, it is important to understand how cell division and apoptosis are modulated, and the identification of molecules that could potentially regulate these processes would be of great clinical value.

Zinc is an essential trace element required for growth (MacDonald, 2000). It is also regarded as a physiological regulator of apoptosis (Truong-Tran et al., 2001). Zinc has been

found to be intricately involved in the complex apoptosis pathway, either by contributing structurally to the stability of apoptotic regulatory factors, or by interacting with effectors that modulate apoptosis (Verhaegh et al., 1998; Smith et al., 2002). Most of the cellular zinc in the body is very tightly bound to macromolecules and is not readily exchangeable. However, there exists a labile intracellular pool of zinc (LIPZ) that is readily exchangeable and more dynamic. Hence, it is believed that LIPZ imparts a more active role in regulating apoptosis (Chai et al., 1999).

Apoptosis is a complex process, and the precise mechanism by which it is controlled is still not clear. Although many studies in the literature have reported a linkage between zinc and apoptosis, the relationship between the dynamic LIPZ and apoptosis has yet to be established for breast cancer. Previous studies from our lab showed an association between tissue zinc accumulation and rat mammary tumourigenesis, and the altered zinc level was attributed to altered zinc homeostasis. Given the dynamic role of LIPZ in cellular zinc homeostasis and its involvement in regulating metabolic growth processes such as apoptosis, it is important to know how LIPZ may influence breast cancer cell growth.

1. Literature Review

1.1. Zinc Nutrition

1.1.1. Overview

Zinc (Zn), a group IIB metal, is an essential trace element for humans, and is ubiquitously distributed among cells (Vallee and Falchuk, 1993). Human zinc requirement varies as a function of age, sex, growth stage and physiological state such as pregnancy (Cousins, 1997). On average, the recommended dietary allowance (RDA) of zinc for adults is 8 mg zinc/day for women and 11 mg zinc/day for men (Institute of Medicine, 2001). Animal products, such as red meats, organ meats and seafood, are the richest sources of zinc. Other good dietary sources of zinc include poultry and dairy products. Whole grains and leafy vegetables represent good plant sources of zinc. On the other hand, fruits and vegetables that are high in fibre and phytate are poor sources of zinc due to low zinc bioavailability (Solomons and Cousins, 1984).

Typically, zinc content in the body is maintained at approximately 1.5 - 2.5 g (King et al., 1994). There is a lack of zinc storage in the body, and zinc homeostasis relies on intestinal absorption and excretion. Zinc is present in all organs, with the highest concentrations found in liver, kidney, bone, muscle and skin. Zinc is present in the body predominantly in the Zn^{2+} form and is primarily complexed with macromolecules such as proteins. The total zinc content in the plasma is about 15 μmol/L (~100 μg zinc/100mL), and comprises less than 1% of total body zinc content (Vallee and Falchuk, 1993).

Zinc has many biological functions and is central to a myriad of physiological processes. It plays critical roles in the functional and structural integrity of cells and tissues. Zinc is required for events as diverse as gene expression, DNA/RNA synthesis and sequence recognition, transcriptional regulation, cell proliferation, enzymatic activity, neurotransmission, immune function, and energy metabolism (Vallee and Falchuk, 1993; Cousins, 1997).

1.1.2. Zinc and Pathological Conditions

Zinc is essential for normal development, and severe zinc deficiency has been demonstrated to be teratogenic in all mammalian species. Zinc deficiency produced excessive apoptotic cell death in rat embryos, especially in the premigratory neural crest cells (Rogers et al., 1995). Some associated symptoms of zinc deficiency in humans are growth retardation, anorexia, irregular food intake, and altered development of the skeletal system (Vallee and Falchuk, 1993). Zinc deficiency also affects cell- and antibody-mediated adaptive immune responses, natural killer cell function, and macrophage phagocytosis, leading to increased susceptibility to a variety of diseases (Chandra, 1980; Moulder and Steward, 1989; Bhaskaram, 2002). A decreased dietary zinc intake and consequent loss of efficiency of zinc-dependent DNA repair systems has been postulated to affect certain diseases, such as dementia and cancer (i.e. leukemia) (Vallee and Falchuk, 1993). Prospective cohort studies have showed that elevated levels of chromosome abnormalities in lymphocytes may be predictive of cancer risk (Fenech, 2002). However, there is no experimental support for an established linkage between zinc intake and dementia or cancer. Many zinc-containing transcriptional factors known as zinc-finger proteins, which will be

4

discussed later, are integral to the maintenance of genome stability and are involved in cancer progression (Taylor et al., 2000). In rat glioma C6 cells, a low intracellular zinc status markedly hinders the binding capability of transcriptional factors to proper DNA sequences and leads to improper DNA repair (Ho and Ames, 2002). Also, the DNA repair and cell cycle control functions of zinc-dependent proteins are lost by displacement of zinc by other divalent metals (Cd^{2+} and Ni^{2+}) that are toxic (Hartwig et al., 2002). In terms of zinc toxicity, high levels of zinc intake is toxic as it can adversely affect copper absorption and can impair erythrocyte Cu/Zn superoxide dismutase (SOD) activity (Institute of Medicine, 2001).

1.2. Zinc Homeostasis

1.2.1. Intracellular Zinc

Zinc is the second most abundant trace mineral in the body, and intracellular zinc accounts for about 95-99% of the total body zinc (Cousins, 1997). The nucleus contains approximately 30-40% of cellular zinc, and the cytoplasm and organelles contain approximately 50%. The remaining zinc (10%) is found in the cell membrane (Smeyers-Verbeke et al., 1977). Intracellularly, zinc is either tightly bound to proteins such as zinc metalloenzymes and zinc-finger proteins representing a largely fixed and non-exchangeable pool of zinc, or loosely bound to proteins, lipids and cytoskeletons, which constitute a labile, exchangeable pool of zinc (Chai et al., 1999). In addition, a small amount of intracellular zinc is present in free cation form (Zn^{2+}) or sequestered in vesicles (zincosomes), also contribute to the labile, exchangeable pool of zinc (Cousins, 1997; Chai et al., 1999). The labile, exchangeable pool of zinc also is known as the labile intracellular pool of zinc (LIPZ). The size of this pool of zinc is extremely small. The actual size of LIPZ is not known, but

likely varies with cell type. For example, LIPZ in human leukaemic lymphocytes, rat splenocytes and rat thymocytes is between 14-31 pmol/10^6 cells (Zalewski et al., 1993). Generally, the size of LIPZ in cytoplasm is in picomolar or nanomolar range in many cells, and different techniques or probes used to measure LIPZ provide somewhat different concentrations (Coyle et al., 1994; Beyersmann and Haase, 2001). The precise concentration of LIPZ has yet to be established.

Two membrane-permeable fluorescent probes that are highly selective for zinc have been often used to visualize and measure the LIPZ. These probes are 6-methoxy-8-para-toluenesulfonamido-quinoline (TSQ) and 2-methyl-8-para-toluenesulphonamido-6-quinolyoxy acetic acid (Zinquin). TSQ and Zinquin are weakly fluorescent, but upon binding to zinc, the fluorescence is intensified. The brightness or the intensity of the zinc-dependent fluorescence correlates to LIPZ content. TSQ has mostly been used for the detection of zinc in fixed tissues and cells in histochemical staining procedures (Frederickson et al., 1987; Nasir et al., 1999). In 3T3 Swiss murine fibroblasts, zinc-deprivation either by treating with an intracellular zinc chelator TPEN (N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine) or culturing in zinc-depleted medium, reductions in TSQ fluorescence were accompanied by suppression in DNA synthesis and cell proliferation (Back et al., 1993; Paski and Xu, 2001). TSQ complexes with free Zn^{2+} in a stable 2:1 or 1:1 ratio. Yet, it is unknown whether zinc in the TSQ-Zn^{2+} complex may equilibrate with mobile protein-bound Zn^{2+}. Zinquin, on the other hand, can monitor zinc fluxes and the distribution of free Zn^{2+} and loosely protein bound zinc within cells and zincosomes within intact living cells (Coyle et al., 1994). Having lower stability constants than TSQ-Zn^{2+} complex, Zinquin-Zn^{2+}

complex, in stepwise 1:1 or 2:1 Zinquin-Zn^{2+} forms, prevent interactions of Zinquin with the zinc tightly bound to metalloenzymes and zinc-finger proteins (Zalewski et al., 1993; Kimura and Aoki, 2001). Zinquin has also been used to quantify the size of LIPZ using spectrofluorimetry and to visualize its intracellular localization using fluorescence microscope. Application of these techniques permits assessment of the changes of LIPZ size induced by either a zinc chelator or a zinc ionophore (Zalewski et al., 1993). Recent evidence has increasingly alluded to the significance of the LIPZ, which can be altered by Zn deprivation and supplementation, in influencing critical cellular events, such as apoptosis (Truong-Tran et al., 2000a).

1.2.2. Zinc Transporters and Regulation

In order to maintain an adequate intracellular zinc concentration for various biological functions, cells have evolved efficient influx-efflux transport system to regulated intracellular zinc homeostasis. Although our knowledge of mammalian zinc transport systems is still limited, studies have shown that various zinc transporters are involved in zinc uptake, efflux, compartmentalization, storage and detoxification (Gaither and Eide, 2001a). In mammals, zinc transporters can be grouped into two families: ZIP family (ZRT1-(zinc-regulated transporter) and IRT1 (ion-regulated transporter-like protein) and CDF family (Cation Diffusion Facilitator). The ZIP family proteins, such as hZIP1 and hZIP2, are responsible for zinc uptake into the cytoplasm (Gaither and Eide, 2000 and Gaither and Eide, 2001b). The CDF family proteins, including ZnT1 (Palmiter et al., 1995), ZnT2 (Palmiter et al., 1996a), ZnT3 (Palmiter et al., 1996b), ZnT4 (Huang and Gitchier 1997), ZnT5 (Kambe et al., 2002) and, more recently, ZnT6 (Huang et al., 2002), transport zinc out

of cells or sequester zinc into intracellular locations. The list of zinc transporters continues to grow, and it seems that organisms have evolved elaborate mechanisms in the regulation and maintenance of cellular zinc levels (Gaither and Eide, 2001a).

1.3. Physiological Functions of Zinc

1.3.1. Zinc Metallothioneins

Metallothionein (MT) is a cysteine-rich, non-enzymatic intracellular protein, which binds to seven equivalents of bivalent metal ions such as zinc and copper with high affinity (Palmiter, 1998). Under physiological conditions, MT is mainly bound by zinc ions, and acts as the major intracellular zinc binding and storage protein (Schmidt and Beyersmann, 1999). MT, through the formation of metallothionein-zinc complex, has been regarded as the thermodynamic "sink" for zinc, thereby regulating zinc homeostasis (Maret, 2000). Physiologically, the free ionic zinc concentration is usually maintained at very low concentrations, typically in the picomolar or nanomolar range (Simons, 1991). In controlling the available, metabolically active pool of cellular zinc, the level of MT is closely related to zinc concentration. Zinc, together with other diverse factors including other metals and glucocorticoids, has been shown to affect MT expression and synthesis. High zinc intakes induce metallothionein expression, while low zinc intakes are associated with lower levels of metallothionein (Cousins and Lee-Ambrose, 1992). The MT and T (thionein; apoprotein) couple system has been demonstrated to control the concentration of readily available zinc. When zinc concentrations reach certain, yet to be defined critical levels, the metal-responsive element-binding transcription factor activates the expression of T, hence allowing the sequestration of zinc in MT (Maret, 2000).

It has been shown that MT functions as a zinc donor in zinc-dependent processes important in cellular proliferation. Researchers have found enhanced MT expression in developing organisms, growing cells and various tumours, indicating a role for MT in cellular differentiation (Woo et al., 1997; Studer et al., 1997). In controlling cell proliferation, the subcellular location of MT varies during the course of the cell cycle and ensures the delivery and trafficking of available zinc to zinc-dependent processes. MT is normally located in the cytoplasm, but a translocation of MT from the cytoplasm into the nucleus occurs in the early synthesis (S) phase of the cell cycle has been observed in primary rat hepatocytes and murine 3T3L1 fibroblasts (Tsujikawa et al., 1994; Schmidt and Beyersmann, 1999). The MT nuclear translocation in 3T3L1 cells was concurrent with an increase in the level of total cellular MT, along with a rapid onset of cell proliferation (Schmidt and Beyersmann, 1999). Apostolova et al. (1999) observed similar subcellular relocalization of zinc and MT during myoblast differentiation. MT can also affect the activity of zinc-dependent proteins such that zinc can be reversibly exchanged between MT and zinc finger transcription factors, thereby modulating the DNA binding affinity and repair capability (Zeng et al., 1991; Cano-Gauci and Sarkar, 1996). The critical question of the precise mechanism of MT in regulating intracellular zinc distribution remains elusive. A recent in vitro study reported that in leukemic human monocytic cell line THP-1, zinc depletion induced by the intracellular zinc chelator (TPEN) decreases the expression of MT (Cao et al., 2001). And, the subsequent supplementation of zinc to the culture medium leads to a proportionate increase in MT expression. Nevertheless, the exact functions of Zn-MT also remain unanswered. There is evidence suggesting that MT may act as a free radical

scavenger and participate in a cellular antioxidant defense system (Maret, 2000; Mocchegiani et al., 2001). However, the true physiological functions of MT remain to be elucidated.

1.3.2. Zinc Metholloenzymes

Under physiological conditions, Zn^{2+} is redox inert and is stable in a biological medium whose potential is under constant flux (Butler, 1998). This unique chemical property of zinc makes it highly favourable in biological systems. Zinc lacks ligand-field stabilization energy and can assume multiple coordination geometries (Huheey et al., 1993). The formation of stable coordination to macromolecules and the display of fast, flexible ligand exchange render zinc an adaptable participant in proteins and enzymes that carry out diverse biological functions. In microorganisms, plants, and animals, over 300 zinc metalloenzymes have been identified to require zinc for their functions (Vallee and Falchuk, 1993). They represent over 50 different types of enzymes. Typically, zinc is coordinated to specific amino acid residues such as histidine (His), glutamate (Glu), aspartate (Asp) and cysteine (Cys) in polypeptide chain (McCall et al., 2000). The role of zinc in these metalloenzymes can be categorized into three basic functions: catalytic, coactive and structural (Vallee and Falchuk, 1993). For example, zinc is catalytically required for the activities of DNA and RNA polymerases. To exert its coactive role, zinc atoms located in non-active sites either enhance or diminish the catalytic capabilities of the enzyme through bridge-liganding with zinc atoms in the active sites of the same enzyme. Phospholipase C (Hough et al., 1989), Cu/Zu superoxide dismutase (Cu/Zn SOD), and alkaline phosphatase (Kim and Wyckoff, 1990) are examples for the coactive role of zinc. In its structural role, zinc controls conformational folding and stability in enzymes, such as protein kinase C

(PKC; Kirchgessner et al., 1996) and alcohol dehydrogenase (ADH; Gergel and Cederbaum, 1996).

Patturajan et al. (1999) observed that in human pathogenic yeast, the removal of Zn^{2+} using o-phenanathroline (5 mmol/L) caused loss of enzyme activity of RNA polymerase II. Similarly, the absence of zinc or lack of zinc binding due to mutation prevents the enzyme assembly of multisubunit DNA-dependent RNA polymerases in *Escherichia coli* (Markov et al., 1999). The presence of Zn^{2+}, introduced by Zn^{2+} ionophore pyrithione, induces the activation of cellular responses mediated by PKC in human B-lymphocytes (Forbes et al., 1991). And, the subsequent removal of Zn^{2+} results in the inhibition of PKC-dependent events. In rats, feeding a zinc-deficient diet (1.2 mg/kg diet) for 12 d resulted in a 10% reduction in PKC enzymatic activity (Kirchgessner et al., 1996). When ADH was treated with nitric oxide (NO), the release of zinc was correlated with the inactivation of the enzyme. The release was due the fact that NO reacted with the Cys residues contained within the zinc/thiolate active sites in ADH to prevent zinc binding (Gergel and Cederbaum, 1996).

1.3.3. Zinc-finger Proteins

Zinc-finger proteins, sometimes referred to as gene regulatory proteins, are mostly nuclear transcriptional factors involved in controlling cell differentiation, DNA replication and repair, and apoptosis (Dreosti, 2001). As the name suggests, zinc-finger proteins contain zinc-finger motif, in which zinc binds to His and/or Cys residues in the protein sequence to create finger-like folds. Zinc-finger proteins represent an extremely important protein structural role. These folded motifs are arranged in different combinations, and can

recognize and bind to a large range of DNA sequences. Although originally thought to be exclusively functional in DNA-binding domains within transcription factors, other zinc-finger motifs have been found in hormone receptors (Klug and Schwabe, 1995). The recognized list of nuclear hormone receptors includes glucocorticoid (Freedman et al., 1988) and estrogen receptors (Vallee et al., 1991). The zinc-finger domain present in protein kinases may also assist in the redistribution of zinc during activation of the signal transduction pathway (Klug and Schwabe, 1995). Poly (ADP-ribose) polymerase (PARP) is a zinc-finger protein that is known for its involvement in DNA repair. PARP is a DNA binding enzyme that catalyzes the post-translational poly(ADP-ribosyl)ation of protein. Mice and cells deficient in this protein have compromised DNA repair capacity (de Murcia et al., 1997; D'Amours et al., 1999). Another important zinc transcription factor is p53, a tumour suppressor. It interacts with DNA and functions to regulate gene expression and cell cycle progression (Verhaegh et al., 1998). The list of zinc-finger identified proteins is ever expanding. Current evidence suggests that over 3% (300-700 genes) of the human genome encodes zinc-fingers (Clark and Berg, 1998; Maret, 2001).

1.3.4. Zinc and Cell Proliferation and Growth

Zinc has profound effects on growth, and zinc deficiency has been demonstrated to suppress food intake and induce growth retardation in both animals and humans (MacDonald, 2000). In rats fed zinc-deficient diets (1 mg zinc/kg diet), decreased body weight and feed efficiency were observed in comparison to rats on the pair-fed control diets (Bolze et al., 1987). Force-feeding rats with zinc-deficient diets using gastric tubes failed to correct growth impairment and even led to fatalities (Park et al., 1986). This suggests that

12

the reduction in food intake may serve as a conservation mechanism to limit growth and, therefore, to maintain a constant zinc level in the body. And failure in cell division and differentiation, and disturbances to cell turnovers in rapidly growing tissues has also been observed in zinc-deficient animals (MacDonald, 2000). In vitro, zinc-deficient cells remain in S phase and fail to undergo mitosis in comparison with zinc-sufficient cells (Prasad et al., 1996). The addition of zinc-containing serum to cell cultures enhances the cellular uptake of zinc, while zinc deprivation by metal chelators causes decreased growth and DNA synthesis (Beyersmann and Haase, 2001).

Although the importance of zinc in growth has long been established and well documented, the biochemical basis for its involvement is still not being well understood. Evidence in this area has been scarce and even controversial. Studies in vivo demonstrated that zinc deficiency-induced reduced feed intake and growth reduction were associated with low circulating levels of growth hormone and insulin-like-growth-factor I (IGF-I) in rats (Clegg et al., 1995; Browning et al., 1998). Growth hormone (GH) contains specific zinc-binding sites, and binding of zinc to GH ensures structural and functional stability (Cunningham et al., 1991). Other rat studies showed that dietary zinc deficiency and chelator-induced zinc depletion impairs the release of growth hormone from the pituitary glands, and decreases the circulating GH concentration in serum by 78% (Root et al., 1979; Roth and Kirchgessner, 1994; Roth and Kirchgessner, 1997). IGF-I promotes growth and is involved in cell cycle regulation, especially the transition from G1 to S phase (De Meyts et al., 1994). Serum IGF-I was lower in rats fed a zinc-deficient diet compared with zinc-adequate controls (Cossack 1988; Dorup et al., 1991). Yet, in other studies, serum IGF-I

levels were not affected by zinc deficiency (Bolze et al., 1987; Clegg et al., 1995). Nevertheless, it is important to note that zinc-deficiency induced growth retardation is not due to a lack of hormones or impaired hormonal function. Maintaining serum GH and IGF-I level either through exogenous administration or force-feeding in zinc-deficient rats failed to reverse growth inhibition. The growth retardation was only restored when zinc-replete diets were introduced (Cossack, 1988; Dick et al., 1993; Browning et al., 1998). Cell culture studies showed that zinc mediates the action of IGF-I by enhancing endogenous IGF-I synthesis and stimulating cell division (Yamaguchi and Hashizume, 1994; Matsui and Yamaguchi, 1995; MacDonald et al., 1998). Huang et al. (1999) found that zinc stimulates DNA synthesis in murine fibroblasts. Swiss murine 3T3 cells treated with the extracellular zinc chelator diethylenetriaminepentaacetic acid (DTPA) decreased transcriptional expression of thymidine kinase and impaired thymidine incorporation into DNA (Chester et al., 1989; Chester et al., 1990). Similarly, zinc deprivation achieved by reduced serum concentration and the addition of zinc chelators, (i.e. TPEN and ethylenediaminetetraacetic acid (EDTA)) to culture media, suppressed proliferation of HL-60 human leukemia cells, 3T3 fibroblast cells and HaCaT human keratinocytes (Morimoto et al., 1992; Thornton et al., 1998; Parat 1999).

Another difficulty encountered in the course of the study of zinc and growth is that the effects of zinc are very much animal/tissue/cell specific and zinc concentration dependant. Hence, an optimal zinc level or concentration for supporting growth has yet to be established. Severe zinc deficiency retards growth without observed reduction in tissue (lung and liver) zinc concentration in rats (Hammermueller et al., 1987). Earlier studies

14

highlighted the significance of various zinc homeostatic controls in maintaining zinc level in the events of fluctuations in the dietary zinc supply. In a rat study, for instance, the dietary reduction of zinc was correlated to a reduction in MT-bound zinc to stabilize tissue zinc level (Richards and Cousins, 1975). Recent cell culture studies demonstrated the linkage between LIPZ and growth. 3T3 Swiss murine fibroblasts grown in a zinc-depleted medium (48 h) resulted in the suppression of cell proliferation and DNA synthesis (Paski and Xu, 2001). This suppression concurred with diminished LIPZ as detected by TSQ fluorescence. The supplementation of zinc to the zinc-depleted medium enhanced DNA synthesis and cell proliferation, along with an accompanied increase in LIPZ. LIPZ also plays a role in growth factor-stimulated cell proliferation. Paski and Xu (2002) reported that IGF-I-stimulated DNA synthesis and cell proliferation were associated with elevations in LIPZ in 3T3 cells.

1.4. Cell Death

The cell death program is regulated in complex ways. There are two types of cell death, necrosis and apoptosis or programmed cell death (Allen et al., 1997).

1.4.1. Necrotic Cell Death

Necrosis is a type of cell death, which is characterized by cellular swelling, disruption of cellular organelles, rupture of cellular membranes, lyses of nuclear chromatin into randomly sized fragments, and the eventual removal of cellular debris. The released cellular debris triggers inflammatory responses to clean up the debris by phagocytes or macrophages. Necrosis is an energy-independent process. It occurs in response to injuries by severe

hypoxia, massive trauma, and prominent adenosine triphosphate (ATP) depletion (Allen et al., 1997; Saikumar et al., 1999).

1.4.2. Apoptotic Cell Death

Apoptosis, on the other hand, is a death or suicidal mechanism encoded in the genome of the organism. It is an energy-dependent, non-inflammatory process occurring in both physiological and pathological conditions (Saikumar et al., 1999). Apoptosis is involved in normal embryological development and morphogenesis of life as such required for sculpting the developing embryo, and is required for maintaining cell population control by removing aberrant cells (Miller and Marx, 1998). Moreover, apoptosis also plays important roles in the pathogenesis of diseases. Under normal physiological conditions, cell proliferation and cell death are maintained at equilibrium. However, alterations in the net balance between cell proliferation and cell death are contributing factors in the etiology of diseases. Both excessive and insufficient activation of apoptosis have been implicated in disease pathogenesis. Many neurodegenerative diseases, such as Alzheimer's, Huntington's and Parkinson's diseases have been attributed to abnormally increased loss of post-mitotic cells due to apoptosis (Miller and Marx, 1998, Saikumar et al., 1999). In contrast, insufficient activation of apoptosis has lead to autoimmune disorders (e.g. type I diabetes mellitus) and tumourigenesis (e.g. lymphoma, leukemia and solid tumours). In the instance of malignant tumours, where there is an uncontrolled cell proliferation, insufficient activation of apoptosis may be the culprit. And this has been supported by mutations and aberrant expressions of apoptosis-regulated genes, such as p53 and Bcl-2 family (Saikumar et al., 1999), which will be discussed later on.

Apoptosis is characterized by cell shrinkage, nuclear condensation or pyknotic nucleus, membrane dilation and blebbing, specific DNA fragmentation, formation of membrane bound apoptotic bodies, and membrane changes that eventually lead to phagocytosis of the affected cells (Allen et al., 1997). Apoptosis can be induced by growth factor deficiencies, ionizing radiation, free radical toxicity, metabolic or cell cycle perturbation and death receptor activation, such as triggered by tumour-necrosis factor (TNF) and Fas-ligands (Huppertz et al., 1999; Rath and Aggarwal, 1999). This highly organized progression of the death process is orchestrated and executed by an array of proteins. These proteins function as activators, effectors, or regulators (Saikumar et al., 1999). The process can be regarded as a cascade-like sequence of events, although the precise sequence is still not clear (Huppertz et al., 1999). Early genetic studies on the neomatode *Caenorhabditis elegans* revealed that apoptosis consists of successive stages. Once the cell is committed to undergo apoptosis, the earliest initiation events are blebbing of cell surface and the translocation of phosphotidylserine (PS) from the inner to the outer leaflet of the plasma membrane as a signal to attract macrophages (Martin et al., 1995). The execution of cell death is achieved through the sequential activation of intracellular proteases or caspases leading to DNA fragmentation (Liu et al., 1997). The fragmentation of DNA at specific internucleosomal linker regions results in unique DNA ladders consisting of multiples of 180-200 base pairs (Allen et al., 1997). The subsequent apoptotic bodies (containing DNA fragments) and collapsed nuclear and cell debris are engulfed and degraded by phagocytic cells (Cohen, 1997; Saikumar et al., 1999).

1.4.3. Regulators of Apoptosis

p53 Tumour Suppressor Protein   The p53 tumour suppressor participates in the initiation of the apoptosis cascade in response to DNA damage (Burns and El-Deiry, 1999). The human p53 protein acts as a transcription factor and plays a key role in the cell's response to genotoxic stress such as DNA damaging agents, hypoxia, nucleotide depletion, and oncogene activation. It performs many biochemical functions as well as regulates multiple cellular processes, including cell proliferation and DNA repair. Under normal conditions in most cells and tissues, the p53 is expressed at barely detectable low levels and has a rapid turnover rate with its half-life being on the order of 20-30 min (Wiman, 1997; Burns and El-Deiry, 1999). In response to the aforementioned stressors, the p53 protein level increases, and it accumulates through protein stabilization and initiates the response pathways. The increased DNA binding activity mediates these responses by regulating (either activating or inhibiting) transcription of target genes containing specific DNA binding sites for p53 domains. The p53-responsive genes include the p21$^{Waf/Cip}$, a nuclear factor that acts on cell cycle progression, pro-apoptotic proteins in the Bcl-2 family (e.g. Bax and Bcl-xS), and gadd45 (growth arrest and DNA damage repair factor) (Tang et al., 1998; Burns and El-Deiry, 1999). Although our present knowledge in this area remains unclear, available evidence has shown that there are both transactivation-dependent and -independent functions of p53 in triggering the apoptotic program (Bellamy, 1997). MDA-MB-231 breast cancer cells treated with the isoflavonoid, genistein, (5-30 μmol/L, 24-72 h) inhibited cell growth and induced apoptosis through a p53-independent pathway (Li et al., 1999). The dependence of p53-activated apoptosis may also be animal or cell-specific. Russo and Russo (2000)

reported that rats treated with human chorinoic gonadotropin (hCG) showed a p53-dependent activation of apoptosis with partial dependence on the Bcl-2 family-related genes (Bcl-xL). Human breast epithelial MCF-10F cells and urothelial T24 cells showed different responses to hCG treatment cells (Srivastava et al., 1998). The incubation of hCG (100 IU/mL, 24 h) reduced cell proliferation and elevated the expressions of p53, Bax and p21$^{Waf/Cip}$ in MCF-10F cells, while no changes in gene expressions were observed in T24 human breast epithelial. A recent study found that single-stranded DNA triggered p53-dependent apoptosis in mammalian BALB/c 3T3 cells, and the response was significantly lower in p53-null cells (Nur-E-Kamal et al., 2003).

Bcl-2 Family Members of the Bcl-2 family represent a major class of intracellular regulators or modulators in the apoptosis pathway. They are present in the endoplasmic reticulum, nucleus and outer mitochondrial membranes. Bcl-2 family includes both pro-apoptotic and anti-apoptotic proteins. The best-characterized pro-apoptotic Bcl-2 family members are Bax and Bid, while the well known anti-apoptotic members include Bcl-2 and Bcl-xL (Pellegrini and Strasser, 1999). The antagonistic members of the Bcl-2 family can physically interact with each other by forming heterodimeric or multimeric associations. It appears that the relative proportions of Bcl-2 family suppressors and promoters determine the susceptibility of the damaged cells to undergo apoptosis. The Bcl-2 family has also been implicated in regulating the release of mitochondrial cytochrome c, which in turn activates a subsequent caspase cascade (Nieves-Neira and Pommier, 1999; Waterhouse and Green, 1999).

Caspases   Caspases function as effectors, particularly in the execution phase of the cascade of events ultimately leading to cell death. They are proteolytic enzymes that have the amino acid cysteine (Cys) in their active sites and cleave their target proteins at specific aspartic acid (Asp) residues. Each consists of a large, inactive precursor (a procaspase), which is itself activated by cleavage at Asp by other caspases. Therefore, prompted by adapter proteins transduced upon receipt of potentially apoptotic signals, caspases are activated to initiate sequentially amplified proteolytic cascades to control apoptosis (Cohen, 1997; Janicke et al., 1998). These activations result in the cleavage of critical cellular substrates, including PARP and nuclear lamins, and activate downstream target proteins involved in the breakdown of nuclear membrane, dismantling of the nucleus by DNAs as well as detachment of neighbouring cells to facilitate phagocytic actions (Allen et al., 1997).

1.5. Zinc and Cell Death

Zinc can induce both necrosis and apoptosis, although the effects are dose- and time-dependent, and cell line specific. Litaka et al. (2001) reported that extracellular zinc concentrations at 150 µmol/L or higher induce both necrosis and apoptosis in thyroid cancer cells. Induction of necrosis was dose-dependent up to a zinc concentration of 500 µmol/L, while the level of apoptosis plateaued at a zinc concentration of 200 µmol/L. In another study conducted on prostate carcinoma cells, the addition of zinc (200-500 µmol/L) for 4-24 h induced necrosis, but not apoptosis (Iguchi et al., 1998). Similarly, zinc at 100-300 µmol/L induced necrosis and apoptosis in human T cell lymphoblastic leukemic cells (Hamatake et al., 2000). There is evidence that necrosis and apoptosis are two events that are inter-

convertible at a certain stage. Although the mechanisms by which zinc induces necrosis and apoptosis are still unclear, it is possible that levels of PARP, intracellular ATP concentration and reduced glutathione may serve as switches between the two processes (Nosseri et al., 1994; Palomba et al., 1996; Leist et al., 1997).

1.5.1. Zinc and Apoptosis

The linkage between zinc and apoptosis was first brought to attention by the work of Elmes (1977), in which the author reported the presence of apoptotic cells in the crypt region of the mucosa of the small intestine from zinc-deficient rats (Elmes, 1977). Although this was the first paper to report zinc-deficiency induced apoptosis, the lack of pair-fed controls in the study raised the question of whether the increased apoptosis was a consequence of zinc deficiency per se or reduced feed intake. Later studies in vivo and in vitro provided the supporting evidence of zinc as a regulator of apoptotic cell death. Zinc deprivation, due to either dietary deprivation in rats or to culture of lymphoid and myeloid cells with zinc-deficient (1.4 μmol/L) culture medium, increased apoptosis (Martin et al., 1991; Nodera et al., 2001). DNA fragmentation, a marker of apoptosis, was observed in mouse neuroblastoma cells exposed to >500 μmol/L of extracellular chelator EDTA (ethylene diaminetetraacetic acid; Sakabe et al., 1998). This induction of apoptosis was reversed by addition of exogenous Zn^{2+} (0.1 mmol/L) as $ZnSO_4$. In contrast, zinc supplementation to cell culture media protected cells against apoptosis induced by an array of cell death stimuli. For instance, addition of zinc (500 μmol/L - 5 mmol/L) to culture media inhibited DNA fragmentation and suppressed apoptosis in thymocytes exposed to dexamethasone and γ-irradiation (Sellins and Cohen, 1987). Similarly, Zn^{2+} (500 μmol/L - 1 mmol/L) have also

21

been shown to block apoptosis in fibroblasts and macrophages, T lymphocytes and human leukaemic cells (Waring et al., 1990; Barbieri et al., 1992; McGowan et al., 1994). These levels of zinc are non-physiological, being 10- to 100-fold greater than the concentration of zinc typically found in plasma and tissues. However, more moderate concentrations of zinc (80-200 µmol/L) induce apoptosis in murine thymocytes (Telford and Fraker, 1995). Thus, zinc can both induce and block apoptosis, and it does so in a concentration-dependent manner.

1.5.2. Labile Intracellular Pool of Zinc and Apoptosis

Both animal and human studies have demonstrated that the body is capable of maintaining relatively constant whole body zinc content over a 10-fold fluctuation in dietary zinc intake (Johnson et al., 1993; Kirchgessner, 1993). In maintaining relatively constant tissue zinc levels, the body makes adjustments in gastrointestinal zinc absorption and intestinal endogenous zinc excretion. In the case of severe zinc deficiency in which the cellular function is disturbed, there is only a slight decrease in total cellular zinc content. Hence, a system of tissue and cellular redistribution of zinc may be present for preserving zinc equilibrium (King et al., 2000). As described earlier, most of the cellular zinc is very tightly bound to cellular proteins such as to zinc metalloenzymes and zinc-finger proteins, and is essentially not readily exchangeable. The labile and readily exchangeable LIPZ therefore is more metabolically important. LIPZ is believed to be important in the regulation of cellular events such as apoptosis (Zalewski et al., 1993; Chai et al., 1999; Truong-Tran et al., 2000b).

The increase in apoptosis in vivo could be a direct consequence of a decrease in intracellular zinc in the affected cells (Fraker and Telford, 1997). Studies in vitro indicate that apoptosis can result from a decline in intracellular zinc in the presence of membrane-permeant zinc chelators, such as TPEN (Chai et al., 1999). TPEN is a divalent cation chelator with low affinities for Ca^{2+} and Mg^{2+}, but very high affinities for Zn^{2+} and Fe^{2+}. Typically, the inhibitory concentration of TPEN is ~ 4 mmol/L for an estimated Zn^{2+} concentration of ~100 μmol/L (Shumaker et al., 1998). LIPZ can be monitored and visualized using Zinquin. Typically, Zinquin complexes with zinc in 1:1 or 2:1 ratio with binding constants of 2.7 ± 0.4x10^6 and 11.7 ± 1.9x10^6 Litre/mole, respectively. These affinities are sufficiently lower than typical structural zinc binding constants (10^{12} to 10^{13} Litre/mole) to zinc metalloenzymes (Zalewski et al., 1993). In addition, TPEN has a much higher affinity for zinc than does Zinquin. Therefore, in cells pretreated with TPEN, decreases in Zinquin fluorescence intensities indicate that TPEN is effective in predominately depleting LIPZ (Truong-Tran et al., 2000a). It has been observed that relatively small changes in LIPZ were sufficient to increase apoptotic DNA cleavages in human leukaemic lymphocytes and rat thymocytes (Zalewski et al., 1993). Large increases in DNA fragmentation were induced with short-term TPEN treatment and only a LIPZ decrease of few (~5-10) pmol/10^6 cell. Conversely, increases in LIPZ using the zinc ionophore sodium pyrithione results in suppression of apoptosis. The increase of LIPZ concentration/level to 50-100 pmol/10^6 cells prevented DNA fragmentation. This evidence suggests that a threshold intracellular zinc concentration may exist, below which apoptosis is induced (Truong-Tran et al., 2000b).

1.5.3. Cellular Targets of Zinc in Apoptosis

Apoptosis is a multi-stage event, and zinc is intricately involved in the complex cellular biochemical pathway. As discussed earlier, zinc has been shown to successfully limit the extent of apoptosis induced by cytotoxins. Mechanistic studies have suggested that the anti-apoptotic effect of zinc appears to be mediated by either suppressing some the signaling pathways leading to caspase activation or by directly regulating some of the apoptotic regulators. However, it is important to note that apoptosis is a complex process in which many different physiological anti-apoptotic factors in cells work concertedly to suppress apoptosis. A level of redundancy may be present, such that the increase in the concentration of one factor may be concomitantly compensated by the decrease in concentration of another (Truong-Tran et al., 2001). Zinc is considered as a physiological regulator of apoptosis, but the precise mechanisms involved are largely not known.

The most studied molecular targets of zinc are the caspases. The role of zinc in inhibiting apoptosis was initially thought to be in the late execution phase, mainly as an inhibitor of Ca^{2+}/Mg^{2+} endonucleases, which are responsible for fragmenting DNA into the characteristic ladder patterns (Cohen and Duke, 1984). Later study by Lazebnik et al. (1993) suggested a more sensitive, earlier staged target of zinc is located in the cytosol. This target was later found to be caspase-3 (Lazebnik et al., 1993, Thornberry and Lazebnik, 1998). Structurally, caspases contain Cys residues in their active sites. Zinc has been found to have high affinities for these residues, and thereby can potentially block their enzymatic activities (Chai et al., 1999). Zinc may interact with the sulfhydryl groups of caspases, the group that may be potentially required for catalytic activity (Troung-Tran et al., 2001). Depletion of

LIPZ by TPEN (1-3 μmol/L) for 24 h activated caspase-1 and -3 dependent apoptosis in retinal and cortical cells, respectively (Hyun et al., 2000). In airway epithelical cells, the chelation of zinc with TPEN results in the rapid activation of caspase-3 like activity and markedly enhanced the susceptibility to hydrogen peroxides-induced apoptosis (Truong-Tran et al., 2000b). Human leukemia cells treated with low zinc media (0.5 μmol/L) for 24 h showed a decrease in LIPZ assessed by Zinquin and subsequent activation of caspase-3 (Duffy et al., 2001). In TPEN-treated epithelial, bronchial and alveolar cells, deprivation of LIPZ was followed by activation of caspase-3. The increase in caspase-3 activity was already detectable after 1-2 h of TPEN treatment (up to 25 μmol/L), suggesting that zinc may suppress steps prior to caspase activation (Chai et al., 2000). This finding is in agreement with an earlier study by Takahashi et al. (1996). This study found that zinc did not block the activity of caspase-3 to cleave its cellular substrates, but rather blocked the mechanisms by which caspase-3 is activated from its inactive prozymogen form. Zinc has also been found to target an array of other caspases in the family. The induction of caspases-3, -8, and -9 activations in human peripheral blood T lymphocytes was achieved by TPEN treatment (5-15 μmol/L; Kolenko et al., 2001). Similarly, in human leukemic U937 cells, pretreatment with $ZnCl_2$ (0.3 mmol/L, 10 min) inhibited ricin-induced activation of caspase-3, -6, and -9 (Tamura et al., 2002). Kondoh et al. (2002) recently showed that apoptosis induced by elevated LIPZ in human leukemic HL-60 cells was accompanied by the proteolysis of PARP. As mentioned earlier, PARP is a substrate of caspases, and the cleavage of PARP has often been used to as a measure of caspase activation. The two-fold elevation in LIPZ compared to control, using 25 μmol/L $ZnSO_4$ and 1 μmol/L ionophore pyrithione treatment for 6 h, induced apoptosis as measured by DNA laddering. Interestingly, further LIPZ elevation (50

μmol/L ZnSO$_4$ and 1 μmol/L ionophore pyrithione, 6 h) decreased apoptosis and increased necrosis. Another study reported that exposure of cortical cells to 400 μmol/L zinc for 15 min markedly increased DNA fragmentation and PARP activation and protein level (Kim and Koh, 2002).

The influence of zinc in apoptosis appears at both transcription and translation levels. In human hepatobloastoma cells, a reduction in cellular zinc was associated with an increase in cellular p53 mRNA levels (Reaves et al., 2000). Fanzo et al. (2001) reported that bronchial epithelial cells treated with zinc-deficient media considerably reduced cellular zinc level and resulted in modulation in p53 and its target, such as gadd45. The abundance of p53 and gadd45 mRNA in the zinc-deficient cells were ~100% higher than that in the control cells. In a more recent study on human lymphoma cells exposed to a DNA-damaging drug, a transient intracellular Zn^{2+} rise paralleled an increase in p53 and preceded an increase in p21^{wafl} (Smith et al., 2002). The breaching threshold of engagement in apoptosis and ensuing DNA damage were preceded by a reduction in free intracellular Zn^{2+}. The mean free Zn^{2+} concentration for the control cells was estimated to be ~100 pmol/10^6 cells using the fluorescent probe Zinquin. It was concluded that the loss of Zn^{2+} might reflect the requirement of zinc in binding to enzymes required for nuclear destruction. Zinc may also influence events at the level of protein synthesis since TPEN-induced apoptosis was attenuated by cycloheximide, a protein synthesis inhibitor, in neurons (Ahn et al., 1998). This signified that LIPZ-depletion might trigger apoptosis by affecting upstream steps prior to the activation of death genes. Another possible area of zinc involvement in regulating apoptosis may be to its association with zinc-finger proteins, such as p53. p53 has a complex

tertiary structure and its core DNA-binding domain (amino acid residues 102-292) is stabilized by zinc ions (Burns and El-Deiry, 1999). Verhaegh et al. (1998) showed that exposure of human breast cancer MCF-7 cells to TPEN induced an accumulation of p53 along with an observed decrease in DNA-binding activity. The subsequent removal of TPEN from the medium allowed p53 to refold into its original conformation, followed by an increase in DNA binding, expression of p21 and cell cycle delay in the G1 phase.

There also has been interest in the synergistic relationship between zinc and the level of Bcl-2 family proteins in regulating apoptosis. The Bcl-2 protein family consists of both pro-apoptotic (e.g. Bax) and anti-apoptotic (e.g. Bcl-2) proteins, and zinc ion has been implicated in regulating the ratio of these two antagonistic groups of proteins and hence modulating the susceptibility of damaged cells to undergo apoptotic death (Korsmeyer, 1999; Pellegrini and Strasser, 1999). In hydrogen-peroxide induced human premonocytic cells, zinc supplementation (1 mmol/L) decreased Bax : Bcl-2 ratio and resulted in an increased resistance to undergo apoptosis (Fukamachi et al., 1998). Also, an up-shift in apoptosis-promoting Bax : Bcl-2 ratio was correlated with a transient intracellular Zn^{2+} accumulation (Smith et al., 2002). Members of the Bcl-2 family also interfere with the mitochondrial release of cytochrome c. TPEN (5-15 μmol/L) treatment caused an anti-apoptotic Bcl-2-induced translocation of cytochrome c from the mitochondrial intra-membranous space into the cytosol and subsequently activated apoptosis (Duffy et al., 2001; Kolenko et al., 2001).

Although recent advances in research and methodology have greatly enhanced our understanding of zinc and apoptosis, many areas have yet to be explored. One such area is

the relationship between LIPZ and apoptotic regulators in discrete subcellular pools, including the intranuclear pools (Longin et al., 1997), microtubular and other cytoskeletal pools (Hesketh, 1982; Zalewski et al., 1990), mitochondrial zinc (Untergrasser et al., 2000; Troung-Tran et al., 2000a), intravesicular zinc (Zalewski et al., 1993; Palmiter et al., 1996a), and zinc within the cell membranes (Bettger and O'Dell, 1981).

1.6. Breast Cancer

Breast cancer is the most frequently diagnosed cancer and is one of the leading causes of death in women with cancer in Canada (Canadian Cancer Statistics, 2002). Although the etiology is not established, it has been demonstrated that the development of breast cancer is influenced by many factors such as ionizing radiation, socioeconomic status, endocrinology, familial or genetic factors and diet (Harris et al., 1997). Amongst all breast cancer cases, about 5-10% are associated with hereditary and consequences of mutations in specific genes, such as BRCA-1 and BRCA-2 (Balz et al., 2002). Mutations in the BRCA-1 gene (on chromosome 17q21) and BRCA-2 (chromosome 13q12-13) are associated with a respective 49% and 28% risk developing breast cancer by the age of 50 (Bennet, 1999; Carolin and Pass, 2000). Other genes, such as p53 tumour suppressor, are known to influence the risk of developing breast cancer. For instance, a recent study indicated that deficiency and mutation in p53 cooperates with BRCA1 and BRCA2 in promoting tumourigenesis (Moynahan, 2002).

1.6.1. Zinc and Breast Cancer

Previous studies in humans and animals suggested that zinc has an important role in mammary tumourigenesis (Chakravarty et al., 1986). The depletion of zinc has been shown to suppress tumour growth of implanted mammary carcinoma (Mills et al., 1984). Margalioth et al. (1983) reported that in humans, zinc concentration in breast cancer tissue is higher than that in normal breast tissues. Previous in vivo studies from our lab indicated an accumulation of zinc in rat N-methyl-N-nitrosourea (MNU)-induced rat mammary tumours (Woo and Xu, 2002). The zinc concentration in the mammary tumours was significantly higher (6-19 x) compared to that in the normal mammary glands. And, the higher mammary tumour zinc concentration occurred regardless of the amount of dietary zinc intake. A subsequent study suggests that the accumulation of zinc was attributed to altered zinc homeostasis (Lee et al., 2003). In MNU-induced rat mammary tumours, the transcriptional expression of zinc efflux transporter (ZnT-1) was reduced by 55%. Moreover, the transcriptional expression and protein level of zinc storage protein (metallothionein) were amplified by 1.3 and 3.5 fold, respectively. Given the role of zinc in growth and cell proliferation, the enhanced accumulation zinc may be needed to support the aberrant, rapid cell growth inherent to tumour growth.

1.6.2. Apoptosis and Breast Cancer

Apoptosis contributes to the overall regulation of a cell population and is vital to normal mammary gland development, due to changes in proliferation that occur during the course of the human lifespan, such as at puberty and pregnancy (Anderson, 1999). Dysregulation of apoptosis plays an important role in breast cancer pathogenesis and

progression. Although the mechanisms involved are still largely unclear and often controversial, apoptosis has been suggested in metastatic breast cancer (Dowsett et al., 1999; Krajewski et al., 1999; Kumar et al., 2000). Apoptosis is frequently detected in breast cancer, using light microscopy in histological sections. A high apoptotic index (AI, number of apoptotic cells as a percentage of cells present) in neoplastic tissues and tumours is an indicator of breast cancer invasiveness and poor prognosis (Lipponen, 1999; Srinivas et al., 2002). Bcl-2 positive (Bcl-2+) breast cancer is associated with low AIs, whereas high AIs are related to p53 positive (p53+) breast cancer. Yet, analyses have revealed that Bcl-2+ patients had favorable prognosis and a higher survival rate (Silvestrini et al., 1996; Zhang et al., 1998). Expression of Bcl-2 protein was also found to be associated with estrogen and progesterone receptor positivities (Leek et al., 1994; Pratt et al., 1998). The apoptosis pathway may even be influenced by or dependent on the steroid hormone system. Moreover, the cell proliferation indicator mitotic index (MI) is closely related to AI with a correlation coefficient of 0.767 (Lipponen, 1999). Breast cancer can occur as a result of many different factors, and apoptosis is executed by an array of regulators. Hence, there could be many biological mechanisms leading to such clinical outcome and the activation of apoptosis. As mechanisms mentioned above, expressions of different apoptosis-regulatory genes or proteins lead to different prognoses in breast cancer patients. The identification of various apoptotic proteins involved in breast cancer will be invaluable in optimizing individual therapeutic strategies (Krajewski et al., 1999).

The Bcl-2 family proteins are involved in mammary gland development (Daidone et al., 1999). Since the ratio of pro-apoptotic (e.g. Bax) to anti-apoptotic (e.g. Bcl-2) proteins

are implicated in the apoptotic process, the expressions of Bcl-2 family proteins are integral to breast cancer. In human breast cancer cells, Bcl-2 and Bax are constitutively expressed (Beck et al., 2002). One of many factors leading to breast malignancy is the up-regulation of Bcl-2 gene expression and inhibition of apoptosis (Green and Beere, 1999). Human prolactin (hPRL) has been linked to a higher breast cancer incidence. Recent study of MCF-7 and T47D human breast cancer cells show that exposure to hPRL resulted in an increased Bcl-2 mRNA level (Beck et al., 2002). When MCF-7 cells were exposed to the growth inhibitor, sodium butyrate, apoptosis was induced and the induction was closely linked with lower levels of Bcl-2 mRNA and protein (Mandal and Kumar, 1996). In breast carcinoma, the expression of Bcl-2 also has been associated with of expressions of steroid receptors (Daidone et al., 1999). Using breast cancer specimens from 27 patients, Eguichi et al. (2000) reported that oncogenic and anti-apotototic Bcl-2 is expressed much less in estrogen receptor α (ERα)-negative breast cancers, which show more malignant phenotypes than ERα-positive. The pro-apoptotic Bax mRNA expression was also much less in ERα-negative breast cancers, along with reduced Bcl-2. The reduction of Bax may play important roles in malignant development of breast cancer to acquire estrogen independency and counteracting the reduced Bcl-2 (Eguchi et al., 2000).

Mutations in the p53 gene and increased expression of p53 protein have been noted as determining factors in human breast cancer (Bergh, 1999). Studies in vivo revealed a tight correlation between p53 status and radiation-induced apoptosis in preneoplastic mouse mammary glands in vivo (Medina et al., 1998). In MCF-7 (p53+) human breast cancer cells, p53 became transcriptionally active upon exposure to ionization radiation (IR) and UV

irradiation. Downstream effectors genes, such as p21 and gadd45 were in turn up-regulated, although each of which independently exhibits growth-suppressive activity (Zhao et al., 2000). MCF-7 cells exposed to camptothecin, a topisomerase I inhibitor, resulted in a 10-fold increase in p53/p21 (Wuerzberger et al., 1998). Other cell culture studies have shown that human breast cancer cell lines can undergo apoptosis independent of p53 status. Rishi et al. (1999) reported that a synthetic retinoid acid was capable of inducing apoptosis and elevate gadd45 mRNA levels in three human breast cancer cell lines with different p53 expression, MCF-7 (p53+), MDA-MB-231 (p53-) and MDA-MB468 (p53-). Likewise, apoptotic responses in HBT3477 breast cancer cells to radioimmunotherapy were independent of p53 (Winthrop et al., 1997). It has been suggested that p53-independent apoptosis in breast cancer cells could be achieved via its modulation of Bax : Bcl-2 ratio (Butt et al., 2000; Winthrop et al., 1997).

The activation of apoptosis in breast cancer cell lines is mainly via non-caspase dependent pathways. β-lapachone induced apoptosis in MCF-7 and T47D cell lines through a noncaspase proteolytic cleavage of PARP (Pink et al., 2000). Similarly, in tributyrin-induced MCF-7 growth arrest and apoptosis, there was a transient increase in Bax followed by a caspase-3 independent cleavage of PARP (Heerdt et al., 1999).

1.7. Summary

Growth can be viewed as the net balance between cell proliferation and cell death. Zinc critically influences both cell proliferation and cell deaths. Recent advances reveal that the LIPZ appears to be an important participant in the dynamics of cell growth. Depletion of

the LIPZ is associated with a reduced DNA synthesis and cell proliferation while an increase in cell proliferation is accompanied by an increased LIPZ. Similarly, chelator-induced depletion of the LIPZ promotes apoptosis while increased LIPZ inhibits apoptosis. This intricate involvement of zinc in apoptosis appears to be through its influence on apoptotic regulatory proteins such as p53 and some members in the Bcl-2 family. Expression of these apoptotic regulatory proteins in breast cancer cells is altered in favor of suppressing apoptosis ultimately to promote the survive of breast cancer cells and to counter the effectiveness of chemotherapy. Moreover, in chemical-induced rat mammary tumourigenesis model, zinc homeostasis appears to be altered to promote zinc accumulation, suggesting that zinc is critical to the growth of mammary tumors. Hence, it is possible that the manipulation of zinc, especially LIPZ, could ultimately affects breast cancer growth through its involvement in cell proliferation and cell death.

2. Hypothesis

Based on the available information, the hypothesis for my thesis research was that LIPZ is critical to the growth of human breast cancer cells. Deprivation of LIPZ will reduce cell survival and promote cell death (Figure I.1).
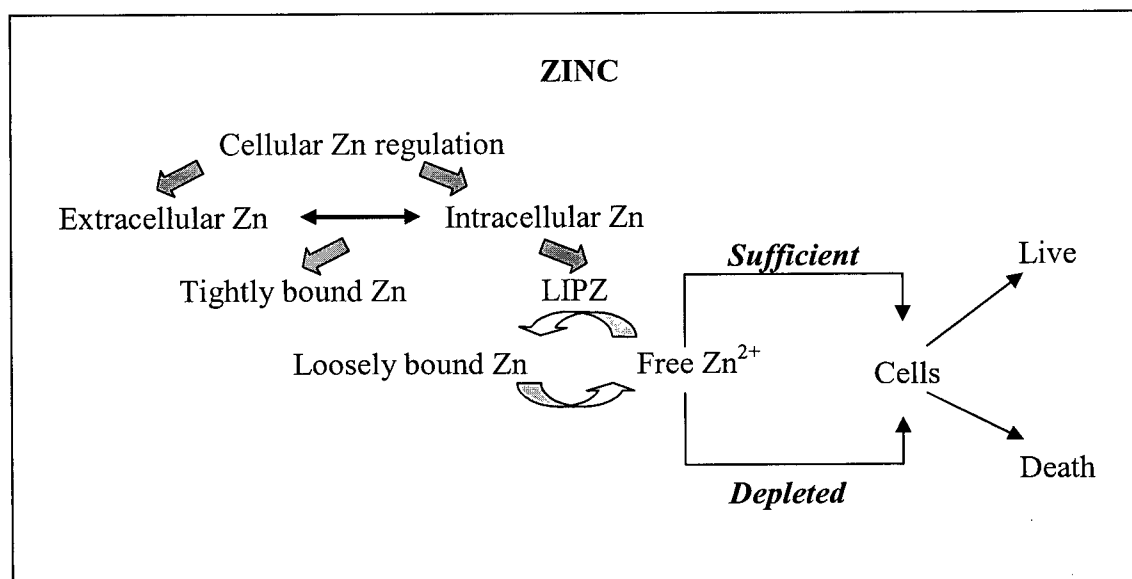
Figure I.1. Hypothesis

3. Objectives

The overall objective of my thesis research project was to investigate the effects of LIPZ depletion on the growth of human breast cancer cells and to explore the possible mechanisms involved. The specific objectives were:

1. To characterize the relationship between LIPZ depletion and growth of human breast cancer cells.

2. To explore the possible mechanism whereby LIPZ influences apoptosis in human breast cancer cells.

4. References

Ahn, Y. H., Kim, Y. H., Hong, S. H. & Koh, J. Y. (1998) Depletion of intracellular zinc induces protein synthesis-dependent neuronal apoptosis in mouse cortical culture. Expt. Neuro. 154: 47-56.

Allen, R. T., Hunter III, W. J. & Agrawal, D. K. (1997) Morphological and biochemical characterization and analysis of apoptosis. J. Pharm. Toxicol. Methods 37: 215-228.

Anderson, T. J. (1999) Pathological studies of apoptosis in the normal breast. Endocr. Relat. Cancer 6: 9-12.

Apostolova, M. D., Ivanova, I. A., & Cherian, M. G. (1999) Metallothionein and apoptosis during differentiation of myoblasts to myotubes: protection against free radical toxicity. Toxicol. Appl. Pharmacol. 159: 175-184.

Back, C., Sisonen, L., Enkvist, M., Heikkila, J. & Akerman K. (1993) Ca^{2+} and Zn^{2+} dependence of DNA synthesis in untransformed and in Ha-ras val-12 expressing NIH 3T3 cells. Exp. Cell. Res. 208: 303-310.

Balz, V., Prisack, H. B., Bier, H. & Bojar, H. (2002) Analysis of BRCA1, TP53, and TSG101 germline mutations in German breast and/or ovarian cancer families. Cancer Genet. Cytogenet. 138: 120-7.

Barbieri, D., Troiano, L., Grassilli, E., Agnesini, C., Cristofalo, E.A., Monti, D., Capri, M., Cossarizza, A. & Franceschi, C. (1992) Inhibition of apoptosis by zinc: a reappraisal. Biochem. Biophys. Res. Commun. 187: 1256-1261.

Beck, M. T., Peirce, S. K. & Chen, W. Y. (2002) Regulation of bcl-2 gene expression in human breast cancer cells by prolactin and its antagonist, hPRL-G129R. Oncogene 21: 5047-5055.

Bellamy, C. O. (1997) p53 and apoptosis. British Med. Bulletin 53: 522-538.

Bennett, L. M. (1999) Breast cancer: genetic predisposition and exposure to radiation. Mole. Carcinog. 26: 143-149.

Bergh, J. (1999) Clinical studies of p53 in treatment and benefit of breast cancer patients. Endocr.-Related Cancer 6: 51-59.

Bettger, W. J. & O'Dell, B. L. (1981) A critical physiological role of zinc in the structure and function of biomembranes. Life Sci. 28: 1425-1438.

Beyersmann, D. & Haase, H. (2001) Functions of zinc in signaling, proliferation and differentiation of mammalian cells. BioMetals 14: 331-341.

Bhaskaram, P. (2002) Micronutrient malnutrition, infection, and immunity: an overview. Nutr. Rev. 60: S40-S45.

Bolze, M. S., Reeves, R. D., Lindbeck, F. E. & Elders, M. J. (1987) Influence of zinc on growth, somatomedin, and glycosaminoglycan metabolism in rats. Am. J. Physiol. 252: E21-E26.

Browning, J. D., Macdonald, R. S., Thornton, W. H. & O'Dell, B. L. (1998) Reduced food intake of zinc deficient rats is normalized by megastrol acetate but not by insulin-like growth factor-I. J. Nutr. 128: 136-142.

Burns, T. & El-Deiry, W. S. (1999) The p53 pathway and apoptosis. J. Cell. Phyl, 181: 231-239.

Butler, A. (1998) Acquisition and utilization of transition metal ions by marine organisms. Science 281: 207-209.

Butt, A. J., Firth, S. M., King, M. A. & Baxter, R. C. (2000) Insulin-like growth factor-binding protein-3 modulates expression of bax and bcl-2 and potentiates p53-independent radiation-induced apoptosis in human breast cancer cells. J. Biol. Chem. 275: 38174-39181.

Canadian cancer statistics (2002). Canadian Cancer Soceity. Toronto, Ontario.

Cano-Gauci, D. F. & Sarkar, B. (1996) Reversible zinc exchange between metallothionein and the estrogen receptor zinc finger. FEBS Lett. 386: 1-4.

Cao, J., Bobo, J. A., Liuzzi, J. P. & Cousins, R. J. (2001) Effects of intracellular zinc depletion on metallothionein and ZIP2 transporter expression and apoptosis. J. Leukocyte Biol. 70: 559-566.

Carolin, K. A. & Pass, H. A. (2000) Prevention of breast cancer. Oncol. Hematol. 33: 221-238.

Chai, F., Troung-Tran, A. Q., Evdokiou, A., Young, G. P. & Zalewski, P. D. (2000) Intracellular zinc depletion induced caspase activation and p21 cleavage in human epithelial cell lines. J. Infect.Diseases 182 (Suppl 1): S85-S92.

Chai, F., Truong-Tran, A. Q., Ho, L. D. & Zalewski, P. D. (1999) Regulation of caspase activation and apoptosis by cellular zinc fluxes and zinc deprivation: a review. Immunol. Cell Biol. 77: 272-278.

Chakravarty, P. K., Ghosh, A. & Chowdhury, J. R. (1986) Zinc in human malignancies. Neoplasia 33: 85-90.

Chandra, R. K. (1980)   Acrodermatitis enteropathica: zinc levels and cell-mediated immunity. Pediatrics 66: 789-791.

Chester, J. K., Petrie, L. & Travis, A. J. (1990) A requirement for Zn^{2+} for the induction of thymidine kinase but not ornithine decarboxylase in 3T3 cells stimulated from quiescence. Biochem.J. 272: 525-527.

Chester, J. K., Petrie, L. & Vint, H. (1989) Specificity and timing of the Zn^{2+} requirement for DNA synthesis in 3T3 cells. Exp. Cell. Res. 184: 499-508.

Clark, N. D., Berg, J. M. (1998) Zinc fingers in Caenorhabditis elegans: finding families and probing pathways. Science 282: 2018-2022.

Clegg, M. S., Keen, C. L. & Donovan, S. M. (1995) Zinc deficiency-induced anorexia influences the distribution of serum insulin-like growth factor-binding proteins in the rat. Metab. Clin. Exp. 44: 1495:1501.

Cohen, G. M. (1997) Caspase: the executioners of apoptosis. Biochem. J. 326: 1-16.

Cohen, J. J. & Duke, R. C. (1984) Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. J. Immunol.132: 38-42.

Cossack, Z. T. (1988) Effect of zinc level in the refeeding diet in previously starved rats on plamsa somatomedin C levels. J. Pediatr. Gastroenterol. Nutr. 7: 441-445.

Cousins, R. & Lee-Ambrose, L. (1992) Nuclear zinc uptake and interactions and metallothionein gene expression are influenced by dietary zinc in rats. J. Nutr. 122: 56-64.

Cousins, R. J. (1997) Zinc. Present Knowledge in Nutrition, 29: 293-306.

Coyle, P., Zalewski, P. D. & Philcox, J. C. (1994) Measurement of zinc in hepatocytes by using a flurosence probe, Zinquin: Relationship to metallothionein and intracellular zinc. Biochem. J. 303: 781-786.

Cunningham, B. C., Mulkerrin, M. G. & Wells, J. A. (1991) Dimerization of human growth hormone by zinc. Science 253: 545-548.

D'Amours, D., Desnoyers, S., D'Silva, I. & Poirier, G. G. (1999) Poly(ADP-ribosyl)ation reactions in the regulation of nuclear function. Biochem. J. 342: 249-268.

Daidone, M. G., Luisi, A., Veneroni, S., Benini, E. & Silverstrini, R. (1999) Clinical studies of Bcl-2 and treatment benefit in breast cancer patients. Endocr.-Related Cancer 6: 61-68.

37

De Meyts, P., Wallach, B., Christoffersen, C. T., Urso, B., Gronskov, K., Latus, L. J., Yakushiji, F., Ilondo, M. M. & Shymko, R. M. (1994) The insulin-like growth factor-I receptor. Structure, ligand-binding mechanism and signal transduction. Horm. Res. 42: 152-169.

de Murcia, J. M., Niedergang, C., Trucco, C., Ricoul, M., Dutrillaux, B., Mark, M., Oliver, F. J., Masson, M., Dierich, A., Lemeur, M., Walztinger, C., Chambon, P. & de Murica G. (1997) Requirement of poly (ADP-ribose) polymerase in recovery from DNA damage in mice and in cells. Proc. Natl. Acad. Sci. U.S.A. 94: 7303-7307.

Dick, D., Rojhani, A. & Cossack, Z. T. (1993) The effect of growth hormone treatment on growth in zinc deficient rats. Nutr. Res. 13: 701-713.

Dorup, I., Flyvbjerg, A., Everts, M. E. & Clausen, T. (1991) Role of insulin-like growth factor-1 and growth hormone in growth inhibition induced by magnesium and zinc deficiencies. Br. J. Nutr. 66: 505-521.

Dowsett, M., Archer, C., Assersohn, L., Gregory, R. K., Ellis, P. A., Salter, J., Chang, J., Mainwaring, P., Boeddinghaus, I., Johnston, S. R. D., Powles, T. J. & Smith, I. E. (1999) Clinical studies of apoptosis and proliferation in breast cancer. Endocr.-Related Cancer 6: 25-28.

Dreosti, I. V. (2001) Zinc and the gene. Mut. Res. 475: 161-167.

Duffy, J. Y., Miller, C. M., Rutschilling, G. L., Ridder, G. M., Clegg, M. S., Keen, C. L. & Daston, G. P. (2001) A decrease in intracellular zinc level precedes the detection of early indicators of apoptosis in HL-60 cells. Apoptosis 6: 161-172.

Eguchi, H., Suga, K., Saji, H., Toi, M., Nakachi, K. & Hayashi ,S. I. (2000) Different expression patterns of bcl-2 family genes in breast cancer by estrogen receptor status with special reference to pro-apoptotic Bak gene. Cell Death and Diff. 7: 439-446.

Elmes, M. E. (1977) Apoptosis in the small intestine of zinc-deficient and fasted rats. J. Path. 123: 219-223.

Fanzo, J. C., Reaves, S. K., Cui, L., Zhu, L., Wu, J. Y. J., Wang, Y. R. & Lei, K. Y. (2001) Zinc status affects p53, gadd45 and c-fos expression and caspase-3 activity in human bronchial epithelial cells. Am.J. Physiol Cell Physiol. 281: C751-C757.

Fenech, M. (2002) Biomarkers of genetic damage for cancer epidemiology. Toxicology 181-182: 411-416.

Forbes, I. J., Zalewski, P. D. & Giannakis, C. (1991) Role of zinc in a cellular response mediated by protein kinase C in human B lymphocytes. Exp. Cell. Res. 195: 224-229.
Fraker, P. J. & Telford, W. G. (1997) A reappraisal of the role of zinc in life and death decisions of cells. Society Exp. Biol. Med. 215: 229-235.

Frederickson, C. J., Kasarskis, E. J., Ringo, D. & Frederickson, R. E. (1987) A quinolline fluorescence method of visualizing and assaying the histochemically reactive zinc in the brain. J. Neurosci. Meth. 20: 91-103.

Freedman, L. P., Luisi, B. F., Korszun, Z. R., Basavappa, R., Sigler, P. B. & Yamamoto, K. R. (1988) The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. Nature, 334: 543-546.

Fukamachi, Y., Karasaki, Y., Sugiura, T., Itoh, H., Abe, T., Yamamura, K. & Higashi, K. (1998) Zinc suppresses apoptosis of U937 cells induced by H_2O_2 through an increase of the bcl-2/bax ratio. Biochem. Biophys. Res. Comm. 246: 364-369.

Gaither, L. A. & Eide, D. J. (2000) Functional characterization of human hZIP2 zinc transporter. J. Biol. Chem. 275: 5560-5564.

Gaither, L. A. & Eide, D. J. (2001a) Eukaryotic zinc transporter and their regulation. BioMetals 14: 251-270.

Gaither, L. A. & Eide, D. J. (2001b) The human ZIP1 transporter mediates zinc uptake in human K56Z erythroleukemia cells. J. Biol. Chem. 276: 22258-22264.

Gergel, D. & Cederbaum, A. I. (1996) Inhibition of the catalytic activity of alcohol dehydrogenase by nitric oxide is associated with S nitrosylation and the release of zinc. Biochem. 35: 16186-16194.

Green, D. R. & Beere, H. M. (1999) Apoptosis and cancer chemotherapy: mechanisms of apoptosis. Hickman J.A. & Dive C. (ed) Humana Press: New Jersey 157-174.

Hamatake, M., Iguchi, K., Hirano, K. & Ishida, R. (2000) Zinc induces mixed types of cell death, necrosis, and apoptosis in Molt4 cells. J. Biochem. 128: 933-939.

Hammermueller, J. D., Bray, T. M., Bettger, W. J. (1987) Effect of zinc and copper deficiency on microsomal NADPH-dependent active oxygen generation in rat lung and liver. J. Nutr. 117: 894-891.

Harris, J., Morrow, M. & Norton, L. (1997) Principles of cancer management: molecular pathology. In: DeVita Jr. VT, Hellman S., Rosenberg, S.A. ed. Cancer: Principles & Practice of Oncology, 5th ed. Lippincott-Raven Publishers, 1557-1570.

Hartwig, A., Asmuss, M., Blessing, H., Hoffmann, S., Jahnke, G., Khandelwal, S., Pelzer, A. & Burkle, A. (2002) Interference by toxic metal ions with zinc-dependent proteins involved in maintaining genomic stability. Food Chem. Toxicol. 40: 1179-84.

Heerdt, B. G., Houston, M. A., Anthony, G. M. & Augenlicht, L. H. (1999) Initiation of Growth Arrest and Apoptosis of MCF-7 mammary carcinoma cells by tributyrin, a

triglyceride analogue of the short-chain fatty acid butyrate is associated with mitochondrial activity. Cancer Research, 59: 1584-1591.

Hesketh, J. E. (1982) Zinc-stimulated microtubule assembly and evidence for zinc binding to tubulin. Int. J. Biochem. 14: 983-990.

Ho, E. & Ames, B. N. (2002) Low intracellular zinc induces oxidative DNA damage, disrupts p53, NFkappa B, and AP1 DNA binding, and affects DNA repair in a rat glioma cell line. Proc. Natl. Acad. Sci. 99: 16770-16775.

Hough, E., Hansen, L. K., Birknes, B., Jynege, K., Hansen ,S., Hordvik, A., Little, C., Dodsen, E. & Derewenda, Z. (1989) High-resolution (1.5A) crystal structure of phospholipase C from Bascillus cereus. Nature 338: 357-60.

Huang, J. S., Mukherjee, J. J., Chung, T., Crilly, K. S. & Kiss, Z. (1999) Extracellular calcium stimulates DNA synthesis in synergism with zinc, insulin and insulin-like growth factor in fibroblast. Eur. J. Biochem. 266: 943-951.

Huang, L. & Gitschier, J. (1997) A novel gene involved in zinc transport is deficient in the lethal milk mouse. Nature 17: 292-297.

Huang, L., Kirschke, C. P. & Gitschier, J. (2002) Functional characterization of a novel mammalian zinc transporter, ZnT6. J. Biol.Chem. 277: 26389-26395.

Huheey, J. E., Koiler, E. A. & Keiter, R. L. (1993) Inorganic Chemistry: Principles of Structure and Reactivity, 4th ed., vol. 1. Harper Collins College Publishers, New York.

Huppertz, B., Frank, H. G. & Kaufmann P. (1999) The apoptosis cascade--morphological and immunohistochemical methods for its visualization. Anatomy and Embryology. 200: 1-18.

Hyun, H. J., Sohn, J., Ahn, Y. H., Shin, H. C., Koh, J. Y. & Yoon, Y. H. (2000) Depletion of intracellular zinc induced macromolecule synthesis- and caspase-dependent apoptosis of cultured retinal cells. Brain Res. 869: 39-48.

Iguchi, K., Hamatake, M., Ishida, R., Usami, Y., Adachi, T., Yamamoto, H., Koshida, K., Uchibayashi, T. & Hirano, K. (1998) Induction of necrosis by zinc in prostate carcinoma cells and identification of proteins associated with this induction. Euro. J. Biochem. 253: 766-770.

Institute of Medicine (2001) Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. National Academy Press, Washington, DC.

Janicke, R. U., Sprengart, M. L., Wati, M. R. & Porter, A. G. (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J. Biol. Chem. 273: 9357-9360.

Johnson, P. E., Hunt, C. D., Milne, D. B. & Mullen, L. K. (1993) Homeostatic control of zinc metabolism in men: zinc excretion and balance in men fed diets low in zinc. Am. J. Clin. Nutr. 57: 557-565.

Kambe, T., Narita, H., Yamaguchi-Iwai, Y., Hirose, J., Amano, T., Sugiura, N., Sasaki, R, Mori K., Iwanaga, T. & Nagao, M. (2002) Cloning and characterization of a novel mammalian zinc transporter, zinc transporter 5, abundantly expressed in pancreatic beta cells. J. Biol. Chem. 277: 19049-19055.

Kim, E. E. & Wyckoff, H. W. (1990) Structure of alkaline phosphatases. Clin. Chim. Acta. 186: 175-187.

Kim, Y. H. & Koh, J. Y. (2002) The role of NADPH oxidase and neuronal nitric oxide synthase in zinc-induced poly(ADP-ribose) polymerase activation and cell death in cortical culture. Exp. Neurol. 177:407-18.

Kimura, E. & Aoki, S. (2001) Chemistry of Zinc (II) flurophore sensors. BioMetals 14: 191-204.

King, J. C., Keen, C. L. Zinc. In: Shils ME, Olson JA, Shike M. eds. Modern nutrition in health and disease, 8th edition. Philadelphia: Lea and Febiger, 1994: 214-230.

King, J. C., Shames, D. M, & Woodhouse, L. R. (2000) Zinc homeostasis in humans. J. Nutr. 130: 1360S-1366S.

Kirchgessner, M. (1993) Homeostasis and hemeorhesis in trace element metabolism. In: Trace Elements in Man and Animals – TEMA 8 (Anke, M., Meissner, D. & Mills, C.F., eds.), pp.4-21, Verlag Media Tourisktik, Dresden, Germany.

Kirchgessner, M., Moser, C. & Roth, H. P. (1996) Activity and subcellular distribution of protein kinase C (PKC) in muscle and brain of force-fed zinc deficient rats. Biol. Trace Element Res. 52: 273-280.

Klug, A. & Schwabe, J. R. W. (1995) Zinc fingers. FASEB J. 9: 597-604.

Kolenko, V. M., Uzzo, R. G., Dulin, N., Hauzman, E., Bukowski, R. & Finke, J. H. (2001) Mechanism of apoptosis induced by zinc deficiency in peripheral blood T lymphocytes. Apoptosis 6: 419-429.

Kondoh, M., Tasaki, E., Araragi, S., Takiguchi, M., Higashimoto, M., Watanabe, Y. & Sato, M. (2002) Requirement of caspase and p38MAPK activation in zinc-induced apoptosis in human leukemia HL-60 cells. Euro. J. Biochem. 269: 6204-11.

Korsmeyer, S. J. (1999) BCL-2 gene family and the regulation of programmed cell death. Cancer Res. 59: 1693-1700.

Krajewski, S., Krajewski, M., Turner, B. C., Pratt, C., Howard, B., Zapata, J. M., Frenkel, V., Robertson, S., Ionov, Y., Yamamoto, H., Perucho, M., Takayama, S. & Reed, J. C. (1999) Prognostic significance of apoptosis regulators in breast cancer. Endocr. Relat. Cancer 6: 29-40.

Kumar, R., Vadlamudi, R. K. & Adam, L. (2000) Apoptosis in mammary gland and cancer. Endocr. Relat. Cancer, 7: 257-69.

Lazebnik, Y. A., Cole, S., Cooke, C. A., Nelson, W. G. & Earnshaw, W. C. (1993) Nuclear events of apoptosis in vitro in cell-free mitotic extracts: a model system for analysis of the active phase of apoptosis. J. Cell Biol. 123: 7-22.

Lee, R., Woo, W., Wu, B., Kummer, A., Duminy, H. & Xu, Z. (2003) Zinc Accumulation in N-Methyl-N-Nitrosourea-Induced Rat Mammary Tumors Is Accompanied by an Altered Expression of ZnT-1 and Metallothionein. Exp. Biol. Med. (Accepted).

Leek, R. D., Kaklamanis, L., Pezzella, F., Gatter, K. C. & Harris, A. L. (1994) Bcl-2 in normal human breast and carcinoma, association with oestrogen receptor-positive, epidermal growth factor receptor-negative tumours and in situ cancer. Brit. J. Cancer 69: 135-139.

Leist, M., Single, B., Castoldi, A. F., Kuhnle, S. & Nicotera, P. (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J. Exp. Med. 185: 1481-1486.

Li, Y., Upadhyay, S., Bhuiyan, M. & Sarkar, H. F. (1999) Induction of apoptosis in breast cancer cells MDA-MB-231 by genistein. Oncogene 18: 3166-3172.

Lipponen, P. (1999) Apoptosis in breast cancer: relationship with other pathological parameters. Endocr. Relat. Cancer, 6: 13-16.

Litake, M., Kakinuma, S., Fujimaki, S., Oosuga, I., Fujita, T., Yamanaka, K., Wada, S. & Katayama, S. (2001) Induction of apoptosis and necrosis by zinc in human thyroid cancer cell lines. J. Endocrin. 169: 417-424.

Liu, X., Zou, H., Slaughter, C. & Wang, X. (1997) DFF, a herterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89: 175-184.

Longin, A. S., Mezin, P., Favier, A. & Verdetti, J. (1997) Presence of zinc and calcium permeant channels in the inner membrane of the nuclear envelope. Biochem. Biophys. Res. Comm. 235: 236-241.

MacDonald, R. S. (2000) The role of zinc in growth and cell proliferation. J. Nutr. 130: 1500S-1508S.

MacDonald, R. S., Wollard-Biddle, L. C., Browing, J. D., Thornton Jr., W. H. & O'Dell, B. L. (1998) Zinc deprivation of murine 3T3 cells by use of diethylenetrinitrilopentaacetate impairs DNA synthesis upon stimulation with insulin-like growth factor-I (IGF-I) J. Nutr. 128: 1600-1605.

Mandal, M. & Kumar, R. (1996) Bcl-2 expression regulates sodium butyrate-induced apoptosis in human MCF-7 breast cancer cells. Cell Growth and Prolif. 7: 311-318.

Mandlekar, S., Yu, R., Tan, T. & Kong, A. N. T. (2000) Activation of caspase-3 and c-Jun NH$_2$-terminal kinase-1 signaling pathways in tamoxifen-induced apoptosis of human breast cancer cells. Cancer Res. 60: 5995-6000.

Maret, W. (2000) The function of zinc metallothionein: a link between cellular zinc and redox state. J. Nutr.130: 1455S-1458S.

Maret, W. (2001) Zinc biochemistry, physiology, and homeostasis – recent insights and current trends. BioMetals 14: 187-190.

Margalioth, E. J., Schenker, J. G. & Chevion, M. (1983) Copper and zinc levels in normal and malignant tissues. Cancer 52: 868-872.

Markov, D., Naryshkina, T., Mustaev, A. & Severinov, K. (1999) A zinc-binding site in the largest subunit of DNA-dependent RNA polymerase is involved in enzyme assembly. Genes Dev. 13: 2439-2448.

Martin, S. J., Mazadai, G., Strain, J. J. Cotter, T. G. & Hannigan, B. M. (1991) Programmed cell death (apoptosis) in lymphoid and myeloid cell lines during zinc deficiency. Clin. Exp. Immunol 83: 338-343.

Martin, S. J., Reutelingsperger, C. P. M., McGahon, A. J., Radar, A., van-Schie, R. C., LaFace, D. M. & Green, D. R. (1995) Early distribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpresion of Bcl-2 and Abl. J. Exp. Med. 182: 1545-1556.

Matsui, T. & Yamaguchi, M. (1995) Zinc modulation of insulin-like growth factor's effect in osteoblastic Mc3T3-E1 cells. Peptides 16: 1063-1068.

McCall, K. A., Huang, C. C. & Fierke, C. A. (2000) Function and mechanism of zinc metalloenzymes. J.Nutr. 130: 1437S-1446S.

McGowan, A. J., Fernandes, R. S., Verhaegen, S. & Cotter, T. G. (1994) Zinc inhibits UV radiation-induced apoptosis but fails to prevent subsequent cell death. Int. J. Radiat. Biol. 66: 343-349.

Medina, D., Stephens, L. C., Bonilla, P. J., Hollmann, A., Schwahn, D., Kuperwasser, C., Jerry, D. J., Butel, J. S. & Meyn, R. E. (1998) Radiation-induced tumourigenesis in preneoplastic mouse mammary glands in vivo: significance of p53 status and apoptosis. Mole. Carcinog. 22: 199-207.

Miller, L. J. & Marx, J. (1998) Apoptosis. Science, 281: 1301-1326.

Mills, B. J., Broghamer, W. L., Higgins, P. J. & Lindeman, R. D. (1984) Inhibition of tumor growth by zinc depletion of rats. J. Nutr. 114: 746-752.

Mocchegiani, E., Muzzioli, M. & Giacconi, R. (2001) Zinc, metallothioneins, immune responses, survival and ageing. Biogerontology. 1: 133-143.

Morimoto, S., Shiraishi, T., Fukuo, K., Koh, E., Kitano, S., Yasuda, O., Tamatani, M. & Ogihara, T. (1992) EDTA induces differentiation and suppresses proliferation of promyelocytic leukemia cell line HL-60--possible participation of zinc. Biochem. Intl. 28: 313-321.

Moulder, K. & Steward, N. W. (1989) Experimental zinc-deficiency effects on cellular responses and the affinity of humoral antibody. Clin. Exp. Immunol. 77: 269-274.

Moynahan, M. E. (2002) The cancer connection: BRCA1 and BRCA2 tumor suppression in mice and humans. Oncogene 21: 8994-9007.

Nasir, M. S., Fahrni, C. J., Suhy, D. A., Kolodsick, K. J., Singer, C. P. & O'Halloran, T. V. (1999) The chemical cell biology of zinc: structure and intracellular fluorescence of a zinc-quinolinesulfonamide complex. J. Biol. Inorg. Chem 4: 775-783.

Nieves-Neira, W. & Pommier, Y. (1999) Apoptotic response to camptothecin and 7-hydroxystaurosporine (UCN-01) in the 8 human breast cancer cell lines of the NCI anticancer drug screen: multifactorial relationships with topoisomerase I, protein kinase C, Bcl-2, p53, MDM-2 and caspase pathways. Int. J. Cancer. 82: 394-404.

Nodera, M., Yangisawa, H.. & Wada, O. (2001) Increased apoptosis in a variety of tissues of zinc-deficient rats. Life Sci. 69: 1639-1649.

Nosseri, C., Coppola, S. & Ghibelli, L. (1994) Possible involvement of poly(ADP-ribosyl) polymerase in triggering stress-mediated apoptosis. Exp. Cell. Res. 212: 367-373.

Nur-E-Kamal, A., Tsai-Kun, L., Zhang, A., Qi, H., Hars, E. & Liu, L.F. (2003) Single-stranded DNA induces ATM/p53-dependent DNA damage and apoptotic signals. J. Biol. Chem. 278: 12475-12481.

Palmiter, R. D., Cole, T. B. & Findley, S. D. (1996a) A mammalian protein that confers resistance to zinc by facilitating vesicular sequestration. EMBO 15: 1784-1791.

Palmiter, R. D., Cole, T. B., Quaife, C. J. & Findley, S. D. (1996b) ZnT-3, a putative transporter of zinc into synaptic vesicles. Proc Natl. Acad. Sci 93: 14934-14939.

Palmiter, R. D. (1998) The elusive function of metallothioneins. Proc. Natl. Acad. Sci. 95: 8428-8430.

Palmiter, R. D. & Findley, S. D. (1995) Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. EMBO 14: 639-649.

Palomba, L., Sestili, P., Cattabeni, F., Zaai, A. & Cantoni, O. (1996) Prevention of necrosis and activation of apoptosis in oxidatively injured human myeloid leukemia U937 cell. FEBS Lett. 390: 91-94.

Parat, M. O., Richard, M. J., Meplan, C., Favier, A. & Beani, J. C. (1999) Impairment of cultured cell proliferation and metallothionein expression by metal chlelator NNN'N'-tetrakis-(2-pyridylmethyl)ethylenediamine. Biol. Trace. Elem. Res. 70: 51-68.

Park, J. H., Grandjean, C. J., Antonson, D. L. & Vanderhoof, J. A. (1986) Effects of isolated zinc deficiency on the composition of skeletal muscle, liver and bone during growth in rats. J. Nutr. 116: 610-617.

Paski, S. C. & Xu, Z. (2001) Labile intracellular zinc is associated with 3T3 cell growth. J. Nutr. Biochem. 12: 655-661.

Paski, S. C. & Xu, Z. (2002) Growth factor stimulated cell proliferation is accompanied by an elevated labile intracellular pool of zinc in 3T3 cells. Can. J. Physiol. Pharmacol. 80: 790-795.

Patturajan, M., Sevugan, M. & Chatterji, D. (1999) DNA-dependent RNA polymerase II from Candida species is a multiple zinc-containing metalloenzyme. IUBMB Life 48: 163-8.

Pellegrini, M. & Strasser, A. (1999) The portrait of the bcl-2 protein family: life, death and the whole picture. J. Clin. Immuno., 19: 365-377.

Pink, J. J., Wuerzerger-Davis, S., Tagliarino, C., Planchon, S. M., Yang, X., Froelich, C. J. & Boothman, D. A. (2000) Activation of a cysteine protease in MCF-7 and T47D breast cancer cells during beta-lapachone-mediated apoptosis. Expt. Cell Res. 255: 144-155.

Prasad, A. S., Beck, F. W., Endre, L., Hanschu, W., Kukuruga, M. & Kumar, G. (1996) Zinc deficiency affects cell cycle and deoxythymidine kinase gene expression in HUT78 cells. J. Lab. Clin. Med. 128: 51-60.

Pratt, C., Krajewski, S., Menard, M., Krajewska, M, MacCleod, H. & Reed, J. (1998) Estrogen withdrawal-induced human breast cancer tumor regression in nude mice is prevented by Bcl-2. FEBS Lett. 440: 403-408.

Rath, P. C. & Aggarwal, B. B. (1999) TNF-induced signaling in apoptosis. J. Clin. Immuno. 19(6): 350-364.

Reaves, S. K., Fanzo, J. C., Arima, F. K., Wu, J. Y. J., Wang, Y. R. & Lei, K. Y. (2000) Expression of the p53 tumor suppressor gene is up-regulated by depletion of intracellular zinc in HepG2 cells. J. Nutr. 130: 1688-1694.

Richards, M. P. & Cousins, R. J. (1975) Mammalian zinc homeostasis: requirement for RNA and metallothionein synthesis. Biochem. Biophys. Res. Commun. 64: 1215-1223.

Rishi, A. K., Sun, R. J., Gao, Y., Hsu, C. K. A., Gerald, T. M., Sheikh, M. S., Dawson, M. I., Reichert, U., Shroot, B., Fornace Jr., A. J., Brewer, G. & Fontana, J. A. (1999) Post-transcriptional regulation of the DNA damage-inducible gadd45 gene in human breast carcinoma cells exposed to a novel retinoid CD437. Nucleic Acids Res. 27: 3111-3119.

Rogers, J. M., Taubeneck, M. W., Daston, G. P., Sulik, K. K., Zucker, R. M., Elstein, K. H., Jankowki, M. A. and Keen, C. L. (1995) Zinc deficiency causes apoptosis but not cell cycle alterations in organogenesis-stage rat embryos: effect of varying duration of deficiency. Teratology 52: 149-159.

Root, A. W., Duckett, G., Sweetland, M. & Reiter, E. O. (1979) Effects of zinc deficiency upon pituitary function in sexually mature and immature male rats. J. Nutr. 109: 958-964.

Roth, H. P. & Kirchgessner, M. (1994) Influence of alimentary zinc deficiency on the concentration of growth hormone (GH), insulin-like growth factor (IGF-I) and insulin in the serum of force-fed rats. Horm Metab. Res. 26: 404-408.

Roth, H. P. & Kirchgessner, M. (1997) Course of concentration changes of growth hormone, IGF-1, insulin and C-peptide in serum, pituitary and liver of zinc-deficient rats. J. Anim. Phys. Anim. Nutr. 77: 91-101.

Russo, I. H. & Russo, J. (2000) Hormonal approach to breast cancer prevention. J. Cell Biochem. 77: 1-6.

Saikumar, P., Dong, Z., Mikhalilov, V., Denton, M., Weinberg, J.M. & Venkatachalam, M.A. (1999) Apoptosis: definition, mechanisms, and relevance to disease. Am. J. Med. 107: 489-506.

Sakabe, I., Paul, S., Danithong, W. & Shinozawa, T. (1998) Induction of apoptosis in Neuro-2A cells by Zn^{2+} chelating. Cell Struct. & Fcn. 23: 95-99.

Schmidt, C. & Beyersmann, D. (1999) Transient peaks in zinc and metallothionein levels during differentiation of 3T3L1 cells. Arch. Biochem. Biophys. 364(1): 91-98.

Sellins, K. S. & Cohen, J. J. (1987) Gene induction by γ-irradiation leads to DNA fragmentation in lymphocytes. J. Immunol 139: 3199-3206.

Shumaker, D. K., Vann, L. R., Goldberg, M. W., Allen, T. D. & Wilson, K. L. (1998) TPEN, a Zn^{2+}/Fe^{2+} chelator with low affinity for Ca^{2+}, inhibits lamin assembly, destabilizes nuclear architecture and may independently protect nuclei from apoptosis in vitro. Cell Calcium 23: 151-164.

Silvestrini, R., Benini, E., Veneroni, S., Daidone, M. G., Tomasic, G., Squicciarini, P. & Salvadori, B. (1996) P53 and bcl-2 expression correlates with clinical outcome in a series of node-positive breast cancer patients. J. Clin. Onc.14: 1604-1610.

Simons, T. J. (1991) Intracellular free zinc and zinc buffering in human red blood cells. J. Membr. Biol. 123: 63-71.

Smeyers-Verbeke, J., May, C., Drochmans, P. & Massart, D. L. (1977) The determination of Cu, Zn, and Mn in subcellular rat liver fractions. Anal. Biochem. 83: 746-753.

Smith, P. J., Wiltshire, M., Davies, S., Chin, S. F., Campbell, A. K. & Errington, R. J. (2002) DNA damage-induced $[Zn2^+]_i$ transients: correlation with cell cycle arrest and apoptosis in lymphoma cells. Am. J. Physiol. Cell Physiol. 283: C609-C622.

Solomons, N. W. & Cousins, R. J. (1984) Zinc. In Solomons N.W., Rosenberg I. H. (eds), Absorption and malabsorption of mineral nutrients. Alan R. Liss, New York, pp 125-197.

Srinivas, P., Abraham, E., Ahamed, I., Madhavan, M., Vijayalakshmi, N. R., Nair, M. K. & Balaram, P. (2002) Apoptotic index: use in predicting recurrence in breast cancer patients. J. Exp. Clin. Cancer Res. 21:233-238.

Srivastava, P., Russo, J., Mgbonyebi, O. P. & Russo, I. H. (1998) Growth inhibition and activation of apoptotic gene expression by human chorionic gonadotropin in human breast epithelial cells. Anticancer Res. 18: 4003-4010.

Studer, R., Vogt, C. P., Cavigelli, M., Hunziker, P. E. & Kagi, J. H. R. (1997) Metallothionein accretion in human hepatic cells is linked to cellular proliferation. Biochem. J. 328: 63-67.

Takahashi, A., Alnemri, E. S., Lazebnik, Y. A., Fernandes-Alnemri, T., Litwack, G., Moir, R. D., Goldman, R. D., Poirier, G. G., Kaufmann, S. H. & Earnshaw, W. C. (1996) Cleavage of lamin A by Mch2 alpha but not CPP32: multiple interleukin 1 beta-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. Proc. Natl. Acad. Sci. U.S.A. 93: 8395-8400.

Tamura, T., Sadakata, N., Oda, T. & Muramatsu, T. (2002) Role of zinc ions in ricin-induced apoptosis in U937 cells. Toxicol. Lett. 132: 141-51.

Tang, D. G., Li, L., Chopra, D. P. & Porter, A. T. (1998) Extended survivability of prostate cancer cells in the absence of trophic factors: increased proliferation, evasion of apoptosis, and the role of apoptosis proteins. Cancer Res. 58: 3466-79.

Taylor, R. M., Whitehouse, C. J. & Caldecott, K. W. (2000) The DNA ligase III zinc finger stimulates binding to DNA secondary structure and promotes end joining. Nucleic Acids Res. 28: 3558-3562.

Telford, W. & Fraker, P. (1995) Preferential induction of apoptosis in mouse CD4$^+$CD8$^+$αβTCRCD3ε thymocyltes by zinc. J. Cell. Physiol. 164: 259-270.

Thornberry, N. A. & Lazebnik, Y. (1998) Caspases: Enemies within. Science: 281: 1312-6.

Thornton, W. H. Jr., MacDonald, R. S., Wollard-Biddle, L. C., Browning, J. D. & O'Dell, B. L. (1998) Chelation of extracellular zinc inhibits proliferation in 3T3 cells independent of insulin-like growth factor-I receptor expression. Proc. Soc. Exp. Biol. Med. 219: 64-68.

Truong-Tran, A. Q., Carter, J., Ruffin, R. E. & Zalewski, P. D. (2001) The role of zinc in caspase activation and apoptotic cell death. BioMetals 14: 315-330.

Truong-Tran, A. Q., Ho, L. H., Chai, F. & Zalewski, P. D. (2000a) Cellular zinc fluxes and the regulation of apoptosis/gene-directed cell death. J. Nutr. 130: 1459s-1466s.

Truong-Tran, A. Q., Ruffin, R. E. & Zalewski, P. D. (2000b) Visualization of labile zinc and its role in apoptosis of primary airway epithelial cells and cell lines. Am. J. Physiol. Lung Cell Mol. Physiol., 279: L1172-L1183.

Tsujikawa, L., Imai, T., Kakutani, M., Kayamori, Y., Mimura T., Otaki, N., Kimura, M., Fukuyama, R. & Shimizu, N. (1994) Localization of metallothionein in nuclei of growing primary cultured adult rat hepatocytes. FEBS Lett. 283: 239-242.

Untergrasser, G., Rumpold, H., Plas, E., Witkowski, M., Pfister, G. & Beger, P. (2000) High levels of zinc ions induce loss of mitochondrial potential and degradation of anti-apoptotic Bcl-2 protein in in vitro cultivated human prostate epithelial cells. Biochem. Biophys. Res. Commum. 279: 607-614.

Vallee, B. L. & Falchuk, K. H. (1993) The biochemical basis of zinc physiology. Am. Physiol. Soc., 73: 79-118.

Vallee, B. L., Coleman, J. E. & Auld, D. S. (1991) Zinc fingers, zinc clusters, and zinc twists in DNA binding protein domains. Proc. Natl. Acad. Sci. 88: 999-1003.

Verhaegh, G. W., Parat, M. O., Richard, M. J. & Hainaut, P. (1998) Modulation of p53 protein conformation and DNA-binding activity by intracellular chelation of zinc. Mol. Carcinog. 21: 205-214.

Waring, P., Egan, M., Braithwaite, A.,Mullbacher, N. & Siaarda, A. (1990) Apoptosis induced in macrophages and T blasts by the mycotoxin sporodesmin and protection by Zn salts. Int. J. Pharmacol. 12: 445-457.

Waterhouse, N. J. & Green, D. R. (1999) Mitochondria and apoptosis: HQ or High-Security Prison? J. Clin. Immunol. 19: 378-387.

Wiman, K. G. (1997) p53: Emergency brake and target for cancer therapy. Expt. Cell Res. 237: 14-18.

Winthrop, M. D., Denardo, S. J., Muenzer, J. T., Chi, S. G. & Gumerlock, P. H. (1997) p53-independent response of a human breast carcinoma xenograft to radioimmunotherapy. Cancer 80: 2529-2537.

Woo, E. S., Monks, A., Watkins, S. C., Wang, A. S. & Lazo, J. S. (1997) Diversity of metallothionein content and subcellular localization in the National Cancer Institute tumor panel. Cancer Chemother. Pharmacol. 41: 61-68.

Woo, W. & Xu, Z. (2002) Body zinc distribution profile during N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats at various levels of dietary zinc intake. Biol. Trace Elem. Res. 87: 157-169.

Wuerzberger, S. M., Pink, J. J., Planchon, S. M., Byers, K. L., Bornmann, W. G. & Boothman, D. A. (1998) Induction of apoptosis in MCF-7: WS8 breast cancer cells by beta-lapachone. Cancer Res. 58: 1876-1885.

Yamaguchi, M. & Hashizume, M. (1994) Effects of beta-alanyl-L-histidinato zinc on protein components in osteoblastic MC3T3-E1 cells: increase in osteocalcin, insulin-like growth factor-1 and transforming growth factor-beta. Mol Cell. Biochem. 136: 163-169.

Zalewski, P. D., Forbes, I. J. & Betts, W. H. (1993) Correlation of apoptosis with change in intracellular labile zinc using Zinquin. Biochem. J., 296: 403-408.

Zalewski, P. D., Forbes, I. J., Giannakis, C., Cowled, P. A. & Betts, W. H. (1990) Synergy between zinc and phorbol ester in translocation of protein kinase C to cytoskeleton. FEBS Lett. 273: 131-134.

Zeng, J., Heuchel, R., Schaffner, W. & Kagi, J. H. R. (1991) Thionein (apometallothionein) can modulate DNA binding and transcription activation by zinc finger containing factor Sp1. FEBS Lett. 279: 310-312.

Zhang, G., Kimijina, I, Abe, R., Watanabe, T., Kanna, M., Hara, K. & Tsuchiya, A. (1998) Apoptotic index correlated to Bcl-2 and p53 protein expression, histological grade and prognosis in invasive breast cancers. Anticancer Res. 18: 1989-1998.

Zhao, H., Jin, S., Antinore, M. J., Lung, F. D. T., Fan, L. F., Blanck, P., Roller, P., Fornace Jr., J. & Zhan, Q. (2000) The central region of gadd45 is required for its interaction with p21/waf1. Expt. Cell. Res. 258: 92-100.

CHAPTER II

N,N,N',N'-TETRAKIS (2-PYRIDYLMETHYL) ETHYLENEDIAMINE-INDUCED DEPLETION OF THE LABILE INTRACELLULAR POOL OF ZINC RESULTED IN SUPPRESSED GROWTH OF MDA-MD-231 BREAST CANCER CELLS

(A revised version of this chapter is in preparation for the submission to Carcinogenesis.)

INTRODUCTION

Growth can be viewed as the net balance between two complementing processes: cell proliferation and cell death (e.g. apoptosis). Zinc has profound effects on growth, and is critically involved in processes on both ends of the balance. Zinc is structurally and functionally important to a myriad of proteins and enzymes that carry out diverse biological processes, including gene expression. Deficiency of this essential mineral consistently causes growth retardation in both animals and humans (Vallee and Falchuk, 1993; MacDonald, 2000). In cells, zinc-deficiency arrests cells in S phase, and they fail to undergo mitosis compared to zinc-sufficient cells, and hence reduces their rate of proliferation (Prasad et al., 1996). Zinc is also known to regulate apoptotic cell death. It can both induce and block apoptosis in a concentration-dependent manner. For example, zinc deprivation, due to either dietary deprivation in rats or treatment with zinc-deficient culture media in lymphoid and myeloid cells, increases apoptosis (Martin et al., 1991; Nodera et al., 2001). In contrast, addition of zinc (> 500 µmol/L) to the culture media inhibits DNA fragmentation and blocks apoptosis in macrophages, T lymphocytes, thymocytes and human leukaemic cells exposed to cytotoxins or irradiation (Waring et al., 1990; Barbieri et al., 1992; McGowan et al., 1994).

Apoptosis is a multi-stage process that is executed by an array of proteins that functions as activators, effectors, or negative regulators (Saikumar et al., 1999). Zinc is intricately involved in the process through its association with apoptotic regulatory proteins such as zinc-finger protein p53 (a tumour suppressor) and its down-stream effector gadd45 (growth arrest and DNA repair factor) (Burns and El-Deiry, 1999; Fanzo et al., 2001). Zinc is also associated with the Bcl-2 protein family, which consists of both pro-apoptotic (e.g. Bax) and anti-apoptotic (e.g. Bcl-2) proteins (Pellegrini and Strasser, 1999). Zinc has been implicated in regulating the ratio of these two antagonistic groups of proteins in modulating the susceptibility of damaged cells to undergo apoptotic death (Fukamachi et al., 1998; Korsmeyer, 1999).

Although the precise sequence of events is still not clear, the dysregulation of apoptosis results in reduced apoptosis, which has been implicated in tumourigenesis, such as breast cancer. The expression or mutation of some apoptotic regulatory proteins such as the p53 and Bcl-2 family have been used as pathological parameters of breast cancer malignancies (Bergh, 1999; Lipponen, 1999). Moreover, zinc has been shown to accumulate in N-methyl-N-nitrosourea-induced rat mammary tumours (Woo and Xu, 2002; Lee et al., 2003), apparently through an altered zinc homeostasis (Woo and Xu, 2002). Since zinc is required to sustain cell proliferation and growth, an accumulation of zinc in rat mammary tumours suggests that zinc plays a role in mammary tumourigenesis.

Most cellular zinc, both intracellular and extracellular, is bound to proteins. Intracellular zinc is either tightly bound to proteins such as metalloenzymes and zinc-finger

proteins, representing a largely fixed and non-exchangeable pool of zinc, or loosely bound to proteins, lipids and cytoskeletons, which constitute a labile, exchangeable pool of zinc (Chai et al., 1999). In addition, a small amount of intracellular zinc, which is present in free cation form (Zn^{2+}), also contributes to the labile intracellular pool of zinc (LIPZ) (Cousins, 1997; Chai et al., 1999). The subcellular distribution and the size of LIPZ can be assessed and measured using zinc-selective membrane-permeable fluorescent probes, such as 6-methoxy-8-para-toluenesulfonamido-quinoline (TSQ) and 2-methyl-8-p-toluenesulphonamido-6-quinolyoxy acetic acid (Zinquin) (Nasir et al., 1999).

LIPZ appears to be metabolically active as its abundance has been associated with cellular events such as cell proliferation and apoptosis (Truong-Tran et al., 2000). For example, addition of NNN'N'-tetrakis-(2-pyridylmethyl)ethlenediamine (TPEN), a membrane-permeable zinc chelator, to the culture medium results in a suppressed cell proliferation of human keratinocytes (Parat, 1999). Similarly, the proliferation and DNA synthesis in 3T3 fibroblasts grown in zinc-depleted media are suppressed concurrently with diminished LIPZ as detected by TSQ fluorescence. The subsequent supplementation of zinc to the zinc-depleted media enhanced DNA synthesis and cell proliferation and was accompanied by an increase in LIPZ (Paski and Xu, 2001). In lymphocytes, TPEN exposure enhances apoptosis, while supplementation of the LIPZ, using zinc ionophore sodium pyrithione results in suppression of apoptosis (Zalewski et al., 1993).

The objectives of this study were to investigate the effects of TPEN-induced zinc depletion on the growth of human breast cancer MDA-MB-231 cells and to explore the

possible mechanism involved. The findings reported herein show that treating cells with TPEN reduced the size of LIPZ without affecting total cellular zinc concentration. This TPEN-induced LIPZ-depletion suppressed overall cell growth, cell viability and survival, and enhances both apoptotic and necrotic cell death. Moreover, TPEN-induced zinc depletion reduced the mRNA level of p53, gadd45, and Bax, while Bcl-2 mRNA level was not affected, indicating an altered expression of some of the apoptotic regulatory proteins. Together, these observations suggest that TPEN-induced LIPZ depletion suppressed the growth of MDA-MB-231 cells via promotion of both necrotic and apoptotic cell death.

MATERIAL AND METHODS

Cells and cell culture

Human breast carcinoma cell line MDA-MB-231 was obtained from the American Type Culture Collection (Manassas, VA). The MDA-MB-231 cells were cultured in Dulbucco Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, U.S.A.) with L-glutamine (2 mmol/L; Gibco, Grand Island, NY, U.S.A.), penicillin (50 U/L) and streptomysin (50 µg/L) (Gibco, Grand Island, NY, U.S.A.), sodium bicarbonate (1.5 g/L; Sigma, St. Louis, MO, U.S.A), and 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, U.S.A.). The zinc concentration in the medium was 4.8 µmol/L. The initial seeding densities were $1x10^4$ cells/well (96-well microplate), $1x10^5$ cells/T-25 flask and $3x10^6$ cells/T-75 flask. The cells were routinely maintained at 37°C in 5% CO_2.

Depletion of labile intracellular pool of zinc

Cells were grown in the culture media for 3 d to establish exponential growth. The culture media were refreshed prior to TPEN treatment. To deplete LIPZ, TPEN (0, 5, 10 or 20 μmol/L; Sigma, St. Louis. Mo., U.S.A.) was added to the medium to form TPEN-treatment media. The cells then were maintained in the TPEN-treatment media for 1 or 8 h at 37°C in 5% CO_2. For the control, an equivalent volume of dimethyl sulphoxide (DMSO), the solvent used to dissolve TPEN, was added to the culture media in place of TPEN.

Quantification of total cellular zinc content

To determine total cellular zinc content, cells were harvested, pelleted and rinsed three times with phosphate buffered saline (PBS) to remove excess extracellular zinc. Upon final rinsing, a small volume (1 mL) of cells was removed for counting. Cells were then pelleted and subjected to acid digestion with 0.5 mL concentrated nitric acid at room temperature. The acid-digested cell suspensions were then quantitatively transferred to acid-washed 1 mL glass volumetric flasks and brought to the volume with double-deionized water. The digested cell samples were further diluted with 0.1 N nitric acid to an appropriate concentration for the determination of total cellular zinc content using flame atomic absorption spectrophotometer (Atomic Absorption Spectrophotometer, Model 2380, Perkin Elmer, Norwalk, CT, U.S.A.). Total cellular zinc concentration was normalized per 10^6 cells.

Quantification of the size of labile intracellular pool of zinc

The size of LIPZ in the cells was quantitated using Zinquin. Zinquin ethyl ester (Calbiochem, San Diego, CA., U.S.A.) was reconstituted in DMSO and stored as 5 mmol/L stock at − 20°C until used. Upon harvest, the cells were washed three times with PBS to remove extracellular zinc prior to labeling with Zinquin. After the final washing, an aliquot (1 mL) of cells was used for counting cell numbers using the Coulter Counter (Beckman Coulter, Model Z1, Miami, FL, U.S.A.). The cells were then diluted with PBS and transferred into a 96-well dark microplate with 1×10^6 cells/well. The cells then were incubated with Zinquin at a final concentration of 6 μmol/L for 30 min at 37°C in the dark on a thermal shaker (Eppendorf Thermomixer R, Brinkman Instruments, Westbury, NY, U.S.A.). The total volume was 200 μL/well. The intensity of fluorescence was measured using a fluorescence microplate reader (SpectraMAX GEMINI XS, Molecular Devices, Sunnyvale, CA, U.S.A.). The excitation and emission peaks were optimized at wavelengths of 365 nm and 485 nm, respectively. For analysis, background fluorescence and autofluorescence of unloaded cells were obtained and subtracted from the readings from the samples. The size of LIPZ was determined by extrapolation from a standard curve constructed using known concentrations of $ZnSO_4$ (Sigma, St. Louis. MO, U.S.A.), and was expressed as pmol/10^6 cells.

Determination of overall cell growth

Overall cell growth was determined by counting the number of cells. Both trypsinized adherent cells and floating cells were harvested for cell counting. After appropriate dilutions

with PBS, cells numbers were measured using the Coulter Counter. Gating calibrations were set so that cell counter excluded cell debris or oversized particles.

Determination of cell viability

Cell Viability was determined using Vybrant™ MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide; Cell Proliferation Assay Kit; Molecular Probe, Eugene, OR, U.S.A.). For this assay, cells were grown in 96-well cell culture microplates. At the end of the TPEN treatment period, microplates were centrifuged at 300 x g for 5 min to pellet the cells. As per manufacturer's directions, the culture media were carefully removed and replaced with fresh media (100 µL/well) and MTT stock solution (12 mmol/L; 10 µL/well). The cells then were incubated at 37°C in 5% CO_2 for 4 h followed by adding 10% SDS (sodium dodecyl sulfate) dissolved in 10 mmol/L HCl solution (100 µL/well) for an overnight incubation at 37°C in 5% CO_2. Absorbance at 570 nm was determined using a microplate reader (Spectramax Plus 384, Molecular Devices, Sunnyvale, CA, U.S.A.). A negative control consisted of the MTT stock solution (10 µL) and 100 µL of the cell culture medium. The absorbance of this negative control was subtracted from the treatment readings.

Flow cytometric detection of cell survival and death

Cell survival and death were determined using Annexin-V-Fluorescein (Annexin-V-FLUOS) and propidium iodide (PI) binding assay (Annexin-V-FLUOS Staining Kit; Roche, Indianapolis, IN, U.S.A.). As per the manufacturer's directions, cells were washed twice with PBS and pelleted by centrifugation at 200 x g for 5 min. The cell pellets then were

resuspended in 100 μL of Annexin-V-FLUOS and PI labeling solution. The solution was incubated for 15 min at room temperature in the dark. After adding HEPES binding buffer (400 μL), the cells were analyzed by flow cytometry within 30 min. The following controls were used to set up compensation and quadrants for staining controls: unstained cells, cells stained with Annexin-V-Fluorescein or with propidium iodide alone, and cells stained with both the dyes. Flow-cytometric analysis of the labeled cells was performed using a FACScan (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.). Early apoptotic cells were defined as Annexin-V positive and propidium iodide negative, whereas dead cells were propidium iodide positive.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNAs were isolated using the single-step RNA isolation procedure according to manufacturer's protocol (RNA Isolation Kit; Qiagen, Mississauga, ON, Canada). Briefly, RNAs suspended in RNase-free DEPC-treated water were stored at -70°C until analysis. RT-PCR was performed using the ThermoScript RT-PCR System plus Platinum Taq DNA Polymerase (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instruction. Total RNA (2 μg) was used for reverse transcription. The resulting cDNA (4 μL, which is equivalent to 0.4 μg total RNA) was subsequently amplified with PCR (GeneAmp PCR System 2400, Perkin Elmer, Norwalk, CT, USA or Eppendorf Mastercycler Gradient Mastercycler, Brinkman Instruments, Westbury, New York, U.S.A.). PCR conditions used were adopted from conditions described earlier (Winthrop et al., 1997; Zhang et al., 1999; Gautschi et al., 2001) with modifications. Briefly, for p53 and gadd45, after a hot start (95°C/2 min), the samples were amplified at 95°C/30 sec; 58°C/30 sec;

72°C/30 sec followed by a final extension at 72°C/10 min. For bcl-2 and bax, after a hot start (95°C/2 min), the samples were amplified at 95°C/40 sec; 60°C/40 sec; 72°C/2 min followed by a final extension at 72°C/7min. Based on the optimization results, the number of PCR cycles used for p53, gadd45, bcl-2 and bax amplification was 29, 33, 39 and 39, respectively. β2-microglobulin was used as an internal control and co-amplified with each PCR amplification. The sequences for the sense and anti-sense primers are listed in Table II.1. Two negative controls: cDNA blank and primer blank were also included with each PCR amplification. Following quantification of the optical density of the band using Scion Image (Release Beta 4.02, Scion Co., MD, USA), mRNA level of p53, gadd45, bcl-2 and bax was normalized on the optical density of the corresponding, co-amplified β2-microglobulin band.

Statistical analyses

The differences among the treatment means were analyzed by ANOVA followed by Tukey's Honesty Test (The SAS System for Windows Release 6.12). Statistical significance was set up at $p < 0.05$.

RESULTS

Total cellular zinc content

The size of LIPZ in the cells cultured in the control medium was 38 pmol/10^6 cells (Figure II.1). Treating the cells with 5, 10, and 20 μmol/L of TPEN for 1 h resulted in a 27, 40, and 78% reduction of the LIPZ content, respectively, demonstrating a dose-dependent effect of TPEN on the size of LIPZ. Treating the cells with 5 μmol/L of TPEN for 8 h had

no effect on the LIPZ content, but higher TPEN concentrations (10 or 20 µmol/L) resulted in a near total depletion of LIPZ. In contrast, the total cellular zinc content remained unchanged regardless of the TPEN concentration used and the treatment duration (Figure II.2). The total cellular zinc content ranges from 30 to 35 nmol/10^6 cells.

Cell growth and cell viability

The effects of TPEN on cell number were used to assess overall cell growth (Figure II.3). Treating the cells with TPEN at 5 or 10 for 1 h had no effect on overall cell growth compared to the cells culture in the control medium. At a higher concentration of TPEN (20 µmol/L; 1 h), a 15% decrease in overall cell growth was observed. When TPEN incubation was extended to 8 h, treating the cells with 5, 10, and 20 µmol/L of TPEN reduced the overall cell growth by 13, 20, and 27%, respectively.

The MTT reduction assay, which measures metabolically active cells, was used for the determination of cell viability. Similar to its effect on the overall cell growth, treating the cells with TPEN at 5 or 10 µmol/L for 1h also had no effect on cell viability. At a higher concentration (20 µmol/L), TPEN treatment reduced the cell viability by 16% (Figure II.4). When TPEN treatment was extended to 8 h, treating the cells with 10 and 20 µmol/L of TPEN reduced the cell viability by 29 and 37%, respectively.

Cell survival and death

To further characterize the possible cause of a TPEN-induced reduction in overall cell growth, the Annexin V- FLUOS and PI staining assay was used to simultaneously sort the live cells from the dead cells, and the necrotic cells from the apoptotic cells (Figure II.5).

After 1 h, there were about 94% live cells, and 2 and 3 % of necrotic and apoptotic cells in the cells cultured in the control medium. The proportion of live and dead cells was not affected by TPEN treatment. When the TPEN treatment was extended to 8 h, TPEN treatment at higher concentration (20 µmol/L) reduced the number of live cells by 32%, and increased the number of necrotic and apoptotic cells by 15 and 13%, respectively, which represented a 7 and 4 fold increase in the number of necrotic and apoptotic cell deaths compared to the cells cultured in the control medium.

p53, gadd45, bcl-2 and bax mRNA Levels

To elucidate the possible causes of the increased apoptotic cell death resulting from TPEN treatment, the mRNA level of several zinc-related apoptotic regulatory genes was assessed by RT-PCR. TPEN treatment at 20 µmol/L reduced mRNA levels of p53 (Figure II.6), gadd45 (Figure II.7), and bcl-2 (Figure II.8) by 18, 36, and 47%, respectively. The bax mRNA level was not affected (Figure II.9). The bax : bcl-2 ratio in the TPEN treated cells was 2.2, which was 1.8 times of the ratio (1.2) in the cells cultured in the control medium.

DISCUSSION

In this study, several parameters were used to assess the effect of LIPZ on breast cancer cell growth. The treatment with TPEN effectively reduced overall cell growth as measured by cell number. This reduction was accompanied by decreased metabolic activity or viability of the cells, measured by the MTT assay. A further analysis using Annexin V-FLUOS and PI staining indicated that the observed reduction in overall cell growth appeared

61

to be attributed to an increased necrotic and apoptotic cell death, along with a decreased cell survival.

The LIPZ size in MDA-MB-231 breast cancer cells was 20-38 pmol/10^6 cells quantitated by using Zinquin, a membrane-permeable, zinc-specific fluorescent probe. This LIPZ size is consistent with observations using the same probe. For example, the LIPZ size in human leukaemic lymphocytes, and rat splenocytes and thymocytes was reported to be between 14-31 pmol/10^6 cells (Zalewski et al., 1993). A more recent study reported the LIPZ size for human lymphoma cells was ~100 pmol/10^6 cells (Smith et al., 2002). It appears that the LIPZ size is very much cell-type specific. The LIPZ size for MDA-MB-231 reported herein is within the range of reported in the literature for a range of cell types.

TPEN, a membrane-permeable intracellular zinc chelator, was effective in depleting the LIPZ, as evidenced by a dose-dependent decrease in the Zinquin fluorescence as TPEN concentration increased. TPEN has a high affinity for Zn^{2+}. Its typical inhibitory concentration is ~ 4 mmol/L for ~100 µmol/L of zinc (Shumaker et al., 1998). Given that the basal Zn^{2+} concentration in the cell culture medium is ~ 5 µmol/L, TPEN concentrations of 5-20 µmol/L used in this study is fairly low in comparison. The depletion of LIPZ was evident as early as after 1 h of TPEN treatment, and the effects were propagated further after 8 h of TPEN treatment. The lack of change in the total cellular zinc content coupled with a reduced LIPZ size supported concept that TPEN chelated mostly LIPZ. This reduced LIPZ size was associated with decreased cell viability and cell survival, and increased cell death suggesting that adequate LIPZ size is critical to cell integrity.

Apoptosis is a physiological mechanism of cell death that plays an important role in cell population control. Dysregulation of apoptosis has been implicated in the development of diseases including cancers. It is characterized by distinct morphological characteristics such as translocation of phosphotidylserine (PS) from the inner leaflet of the plasma membrane to the outer leaflet of the plasma membrane or PS flip (Martin et al., 1995), formation of apoptotic bodies (Saikumar et al., 1999), and specific internucleosomal DNA fragmentation into unique DNA ladders (Allen et al., 1997). Adequate LIPZ size is important to the integrity of cells. Zalewski et al. (1993) have showed that a TPEN-induced decrease of ~5-10 pmol/10^6 cells in the LIPZ size results in large increases (~10-30%) in the rate of DNA fragmentation. Conversely, an increase in LIPZ size by 50-100 pmol/10^6 cells is sufficient to prevent DNA fragmentation. In our study, treatment of cells with 20 µmol/L TPEN reduced the LIPZ size by about 20 pmol/10^6 cells. Correspondingly, there was a 4-fold increase in apoptosis indicated by the PS flip, an early event in apoptosis. However, it is important to note that the analysis of cell deaths using Annexin-V-FLUOS and PI does not have sufficient sophistication to differentiate late apoptotic cells from necrotic cells. This is because that at the late stage of apoptosis, the cells that have undergone apoptosis have lost their membrane integrity. As a result, the cells can be labeled with both Annexin-V-FLUOS and PI and counted as necrotic cells. Therefore, it is likely that apoptosis was underestimated while necrosis was overestimated.

Although there is evidence linking zinc to breast carcinogenesis, no study to date has reported the influence of zinc on apoptosis in breast cancer. The results reported herein show

an increased apoptosis in LIPZ-depleted MDA-MB-231 cells. In these cells, p53 mRNA level was decreased. Apoptosis can be both p53-dependent and -independent (Bellamy, 1997) in a species, tissue, and cell type-specific manner. In MDA-MB-231 breast cancer cells, the isoflavonoid genistein inhibited growth and induced apoptosis through a p53-independent pathway (Li et al., 1999). The p53 expressed in MDA-MB-231 breast cancer cells has a single mutation at codon 280 (Arg → Lys; Elledge et al., 1995). Perceivably, increased apoptosis observed in this study is p53-independent. Furthermore, it has been showed that p53-independent apoptosis in breast cancer cells could be achieved via its modulation of bax : bcl-2 protein ratio (Winthrop et al., 1997; Butt et al., 2000). The bax : bcl ratio in LIPZ-depelted MDA-MB-231 cells was 1.8 times greater than that in the control cells. Since bax is pro-apoptotic while bcl-2 is anti-apoptotic, an increased bax : bcl-2 ratio represents a pro-apoptotic status in these LIPZ-depleted cells. Therefore, it appears that LIPZ-depletion-induced apoptosis in MDA-MB-231 cells reported herein is p53-independent and regulated by proteins in the Bcl-2 family.

Fanzo et al. (2001) reported increased levels of p53 and gadd45 mRNA in bronchial epithelial cells grown in a zinc-deficient medium (0.4 μmol zinc/L) compared to the level in these cells grown in a normal zinc media. Similarly, p53 mRNA levels were also higher in HepG2 cells grown in zinc-deficient media than in cells grown in zinc-sufficient media (Reaves et al., 2000). In the present study, the abundance of p53 and gadd45 mRNA was lower in LIPZ-depleted MDA-MB-231 cells than in control cells. The mechanisms responsible for this apparent contradiction are not known. However, there are several differences between the system employed in this study and the systems reported in the

literature. First, MDA-MB-231 cells express mutant p53 while bronchial epithelial cells and HepG2 cells express functional p53. Secondly, treating cells with TPEN for 8 h in our system is more acute and severe than culturing cells in zinc-deficient media over several days. Thirdly, expression of gadd45 can also be regulated by a p53-independent mechanism in mammalian cells (Smith and Fornace, 1996). Further studies are warranted to elucidate the mechanisms involved.

In summary, we have observed that LIPZ is critical to the overall growth of human breast cancer MDA-MB-231 cells, such that the deprivation of LIPZ reduces cell viability, survival and promotes both necrotic and apoptotic cell death. The increased apoptotic cell death appeared to involve a p53-independent pathway and proceeded through a Bcl-2 related mechanism. Possible involvement of other apoptotic regulatory factors requires further investigation. The rapid removal of LIPZ via chelation is by no means physiological, and may not mimic biological situations. Nevertheless, this study demonstrates the participatory role of LIPZ in apoptosis in breast cancer.
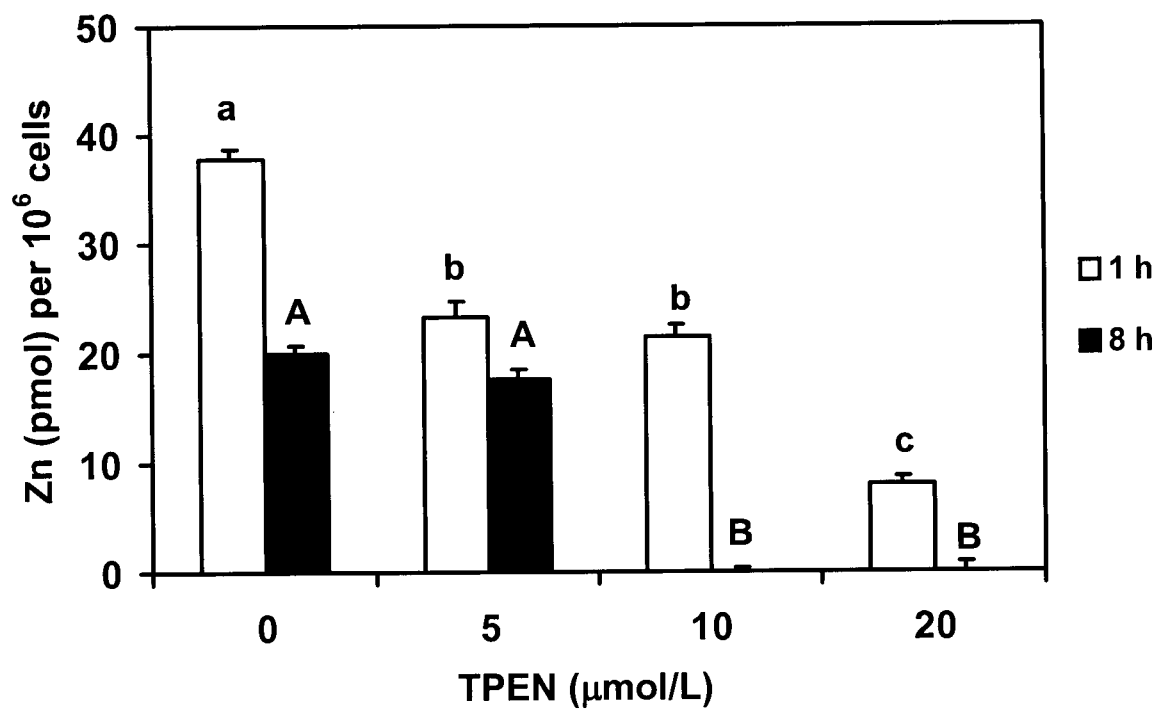
Figure II.1. Effect of TPEN concentration and treatment duration on the LIPZ content in MDA-MB-231 breast cancer cells. Cells were treated with TPEN (0 (DMSO), 5, 10, or 20 μmol/L) for 1 (open bar) or 8 h (filled bar). Values represent mean $\pm$ SEM (n = 4). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).
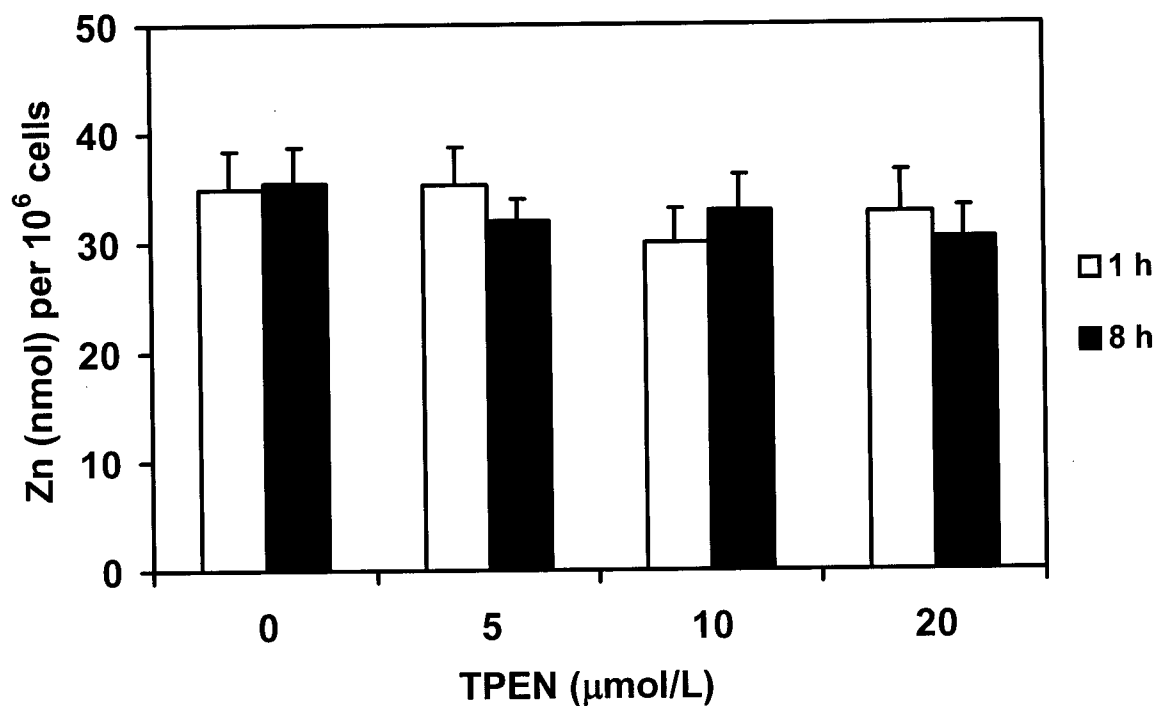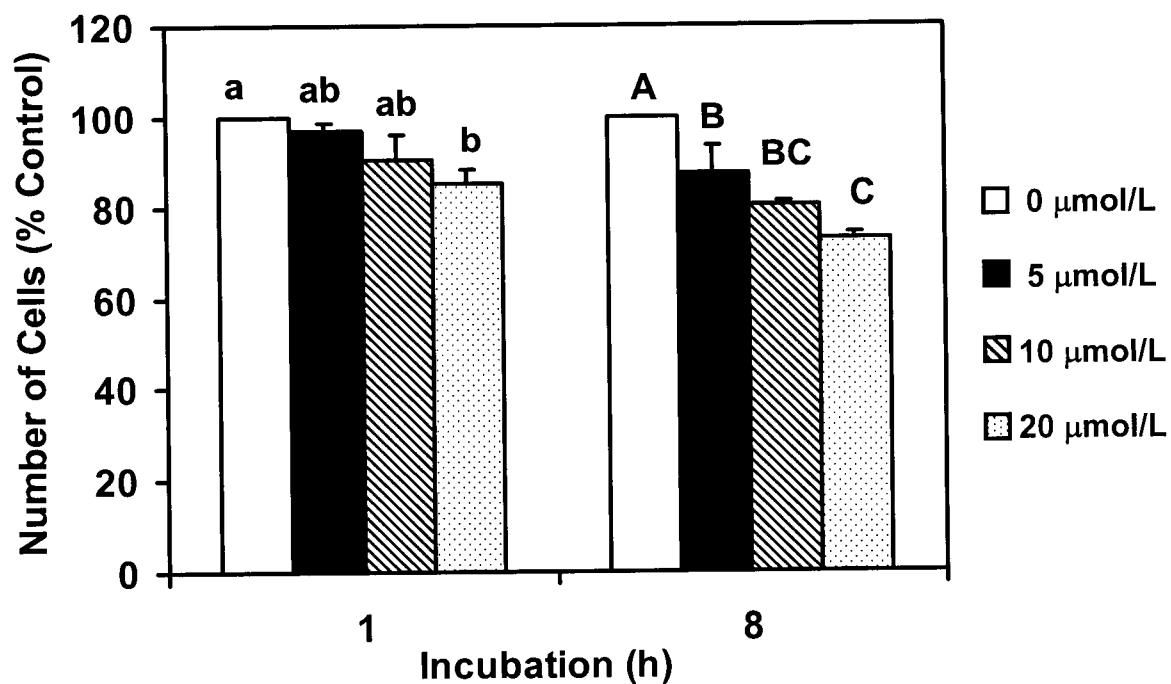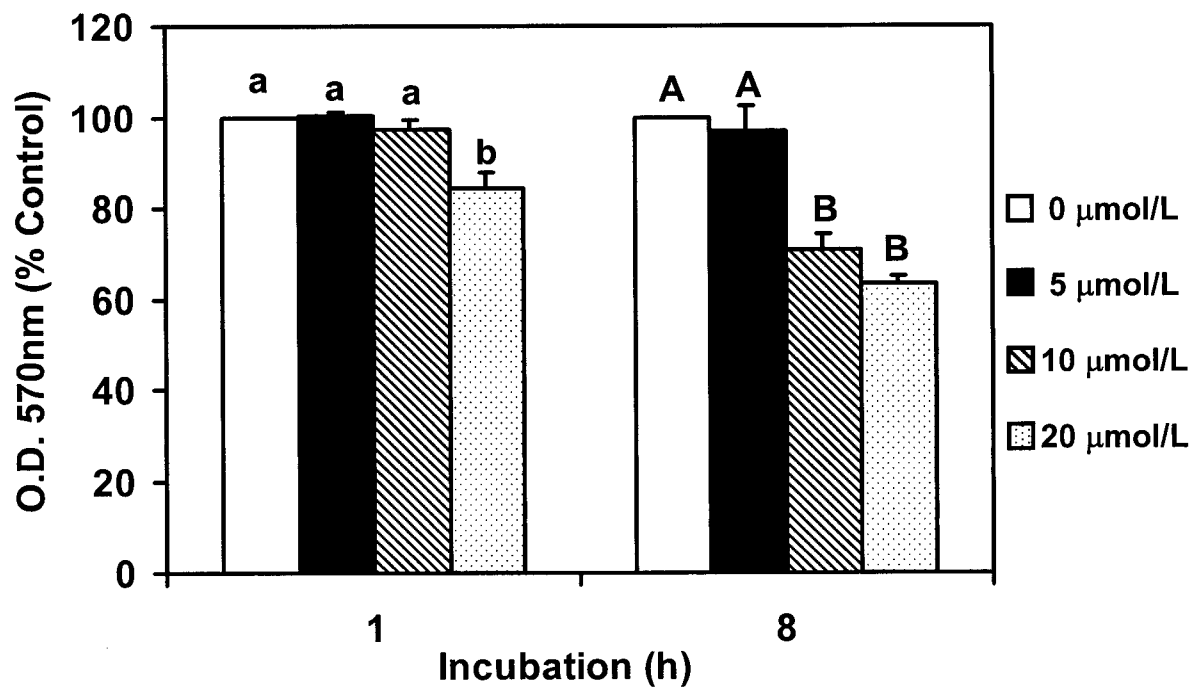
Figure II.2. Effect of TPEN concentration and treatment duration on the total cellular zinc content in MDA-MB-231 breast cancer cells. Cells were treated with TPEN (0 (DMSO), 5, 10, or 20 μmol/L) for 1 (open bar) or 8 h (filled bar). Values represent mean ± SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).
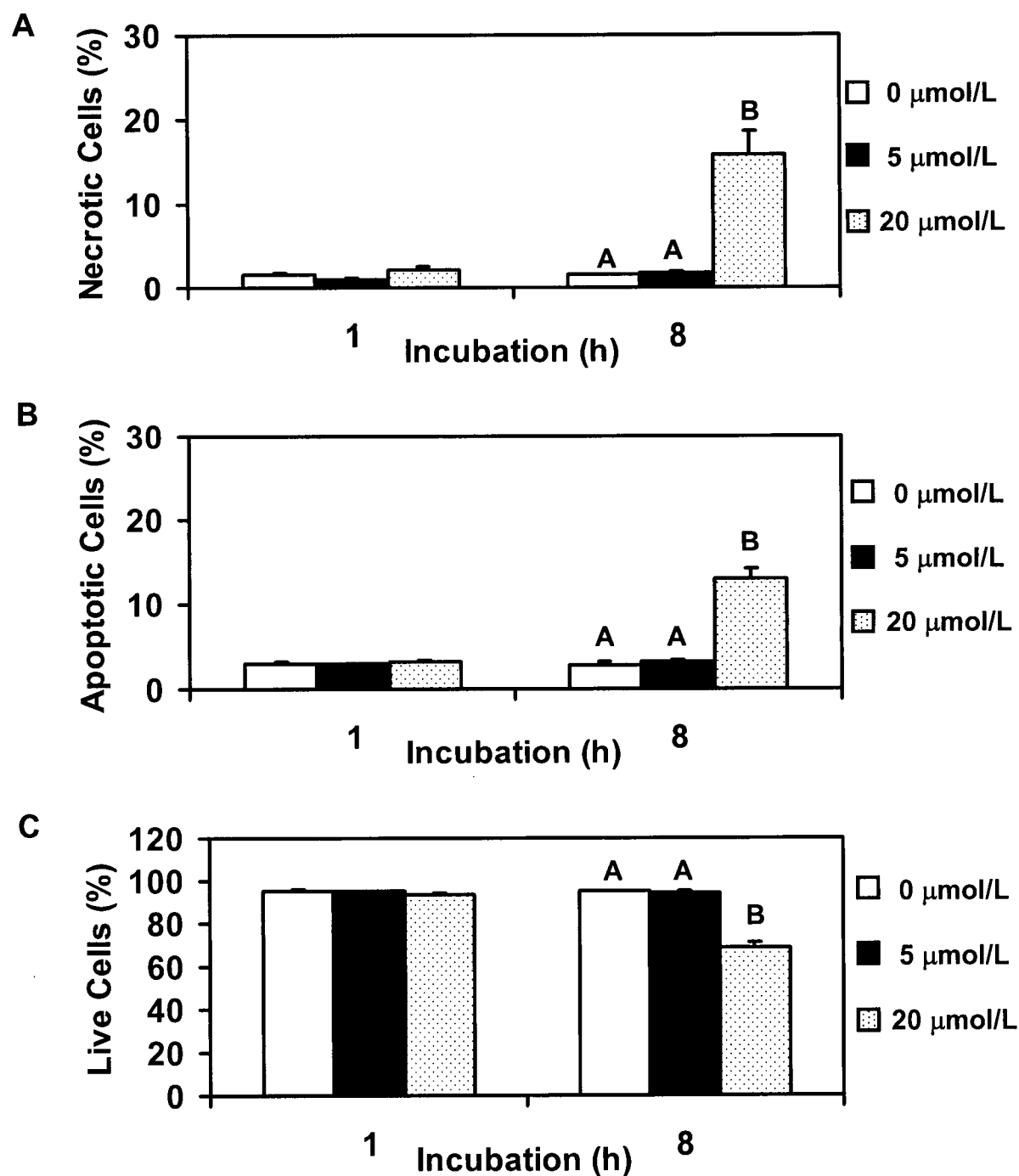
Figure II.3. Effect of TPEN concentration and treatment duration on overall cell growth in MDA-MB-231 breast cancer cells. Cells were treated with TPEN (0 (DMSO) (open bar), 5 (filled bar), 10 (striped bar), or 20 (dotted bar) μmol/L) for 1 or 8 h. Values represent mean ± SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).

Figure II.4. Effect of TPEN concentration and treatment duration on cell viability in MDA-MB-231 breast cancer cells. Cells were treated with TPEN (0 (DMSO) (open bar), 5 (filled bar), 10 (striped bar), or 20 (dotted bar) μmol/L) for 1 or 8 h. Values represent mean $\pm$ SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).

Figure II.5. Effect of TPEN concentration and treatment duration on cell survival and deaths in MDA-MB-231 cells. (A) Necrotic cell death. (B) Apoptotic cell death. (C) Live cells. Cells were treated with TPEN (0 (DMSO) (open bar), 5 (filled bar), or 20 (dotted bar) μmol/L) for 1 or 8 h. Values represent mean ± SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).
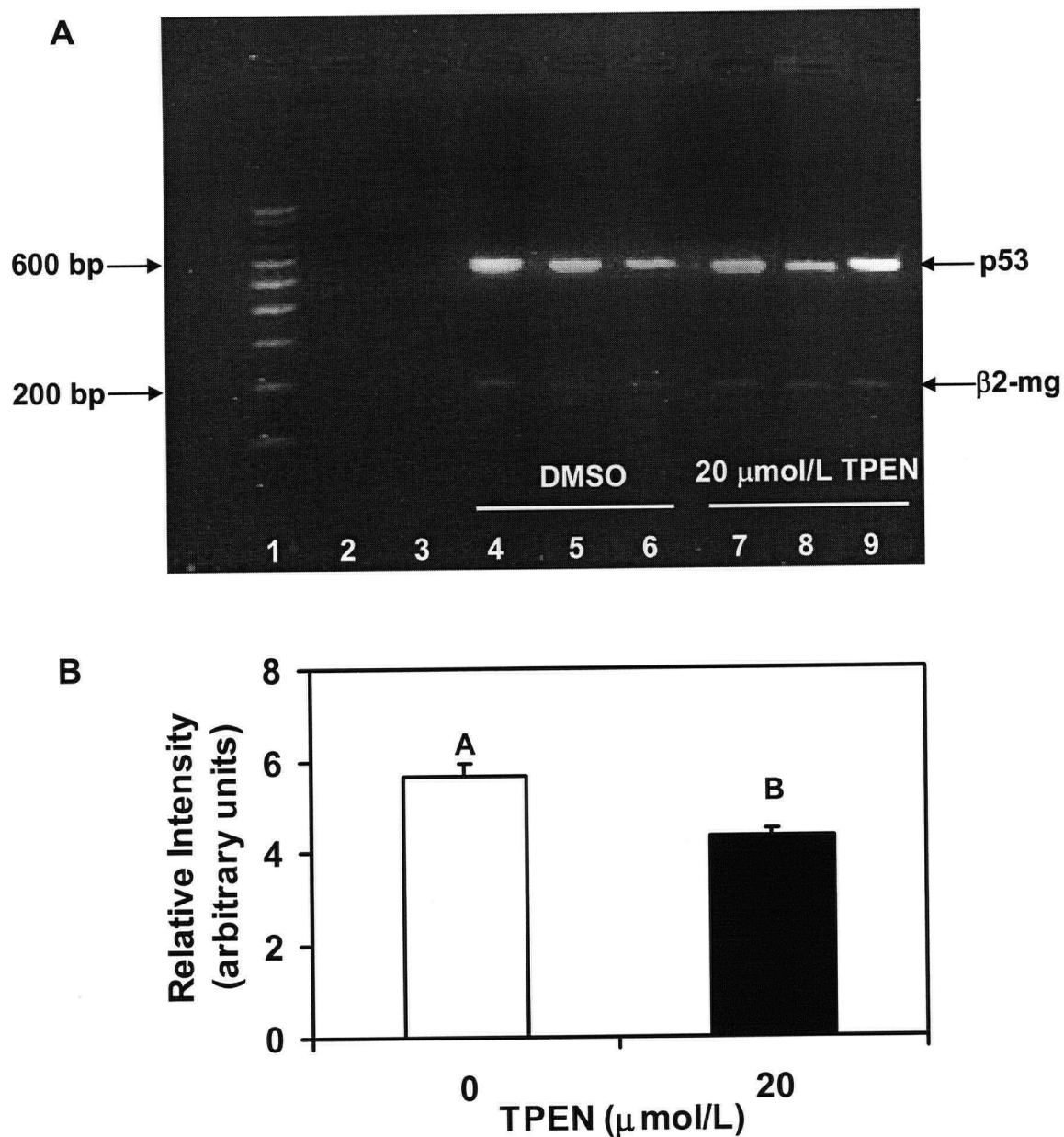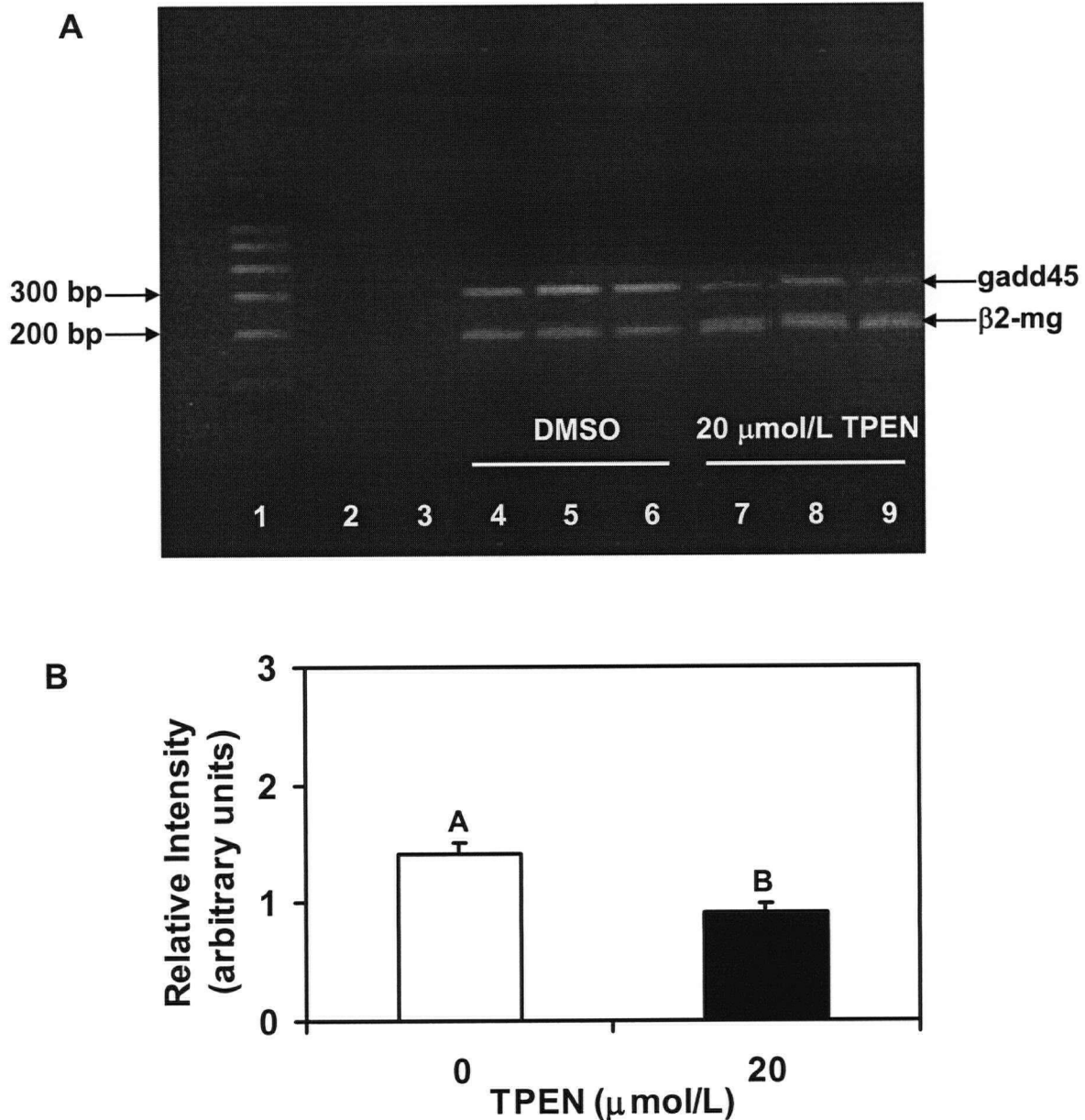
Figure II.6. RT-PCR analysis of p53 mRNA level in MDA-MB-231 cells treated with TPEN for 8 h. Total RNAs were isolated from cells and were reverse transcribed as described in the Material and Methods. PCR products were subjected to agarose (2%) gel electrophoresis. (A) Representative ethidium bromide-stained agarose gel showing the level of p53 and co-amplified (β2-mg) mRNAs. Lane 1: DNA ladder; Lane 2: negative control (no cDNA); Lane 3: negative control (no primers); Lane 4-6: TPEN 0 µmol/L (DMSO); Lane 7-9: TPEN 20 µmol/L. (B) Relative p53 mRNA level normalized on the optical density of corresponding co-amplified β2-microglobuin band. Values represent mean ± SEM (n=5). Means with different letters are significantly different ($p < 0.05$).
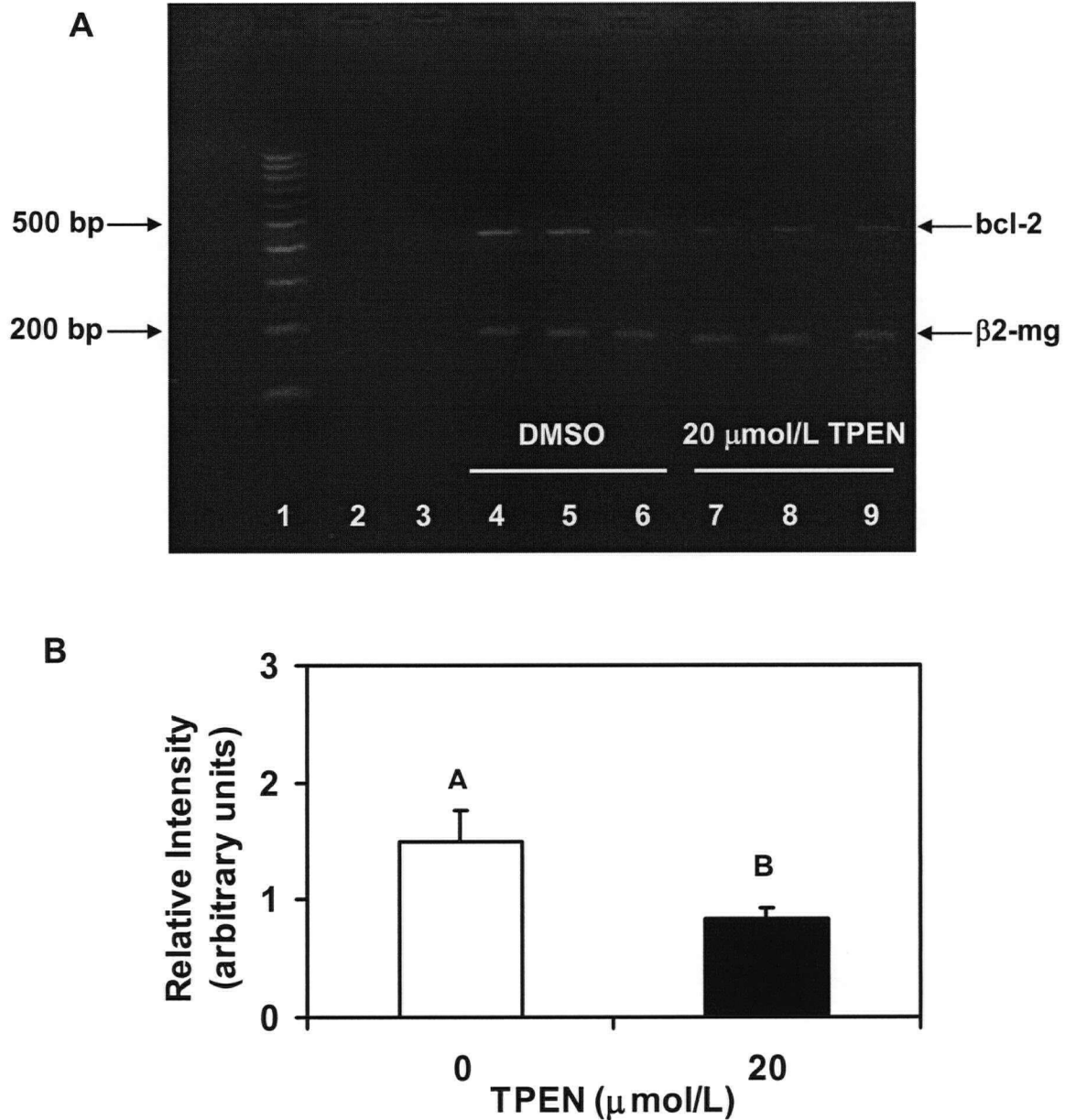
Figure II.7. RT-PCR analysis of gadd45 mRNA level in MDA-MB-231 cells treated with TPEN for 8 h. Total RNAs were isolated from cells and were reverse transcribed as described in the Material and Methods. PCR products were subjected to agarose (2%) gel electrophoresis. (A) Representative ethidium bromide-stained agarose gel showing the level of gadd45 and co-amplified β2-microglobuin (β2-mg) mRNAs. Lane 1: DNA ladder; Lane 2: negative control (no cDNA); Lane 3: negative control (no primers); Lane 4-6: TPEN 0 μmol/L (DMSO); Lane 7-9: TPEN 20 μmol/L. (B) Relative gadd45 mRNA level normalized on the optical density of corresponding co-amplified β2-microglobuin band. Values represent mean ± SEM (n=5). Means with different letters are significantly different ($p < 0.05$).
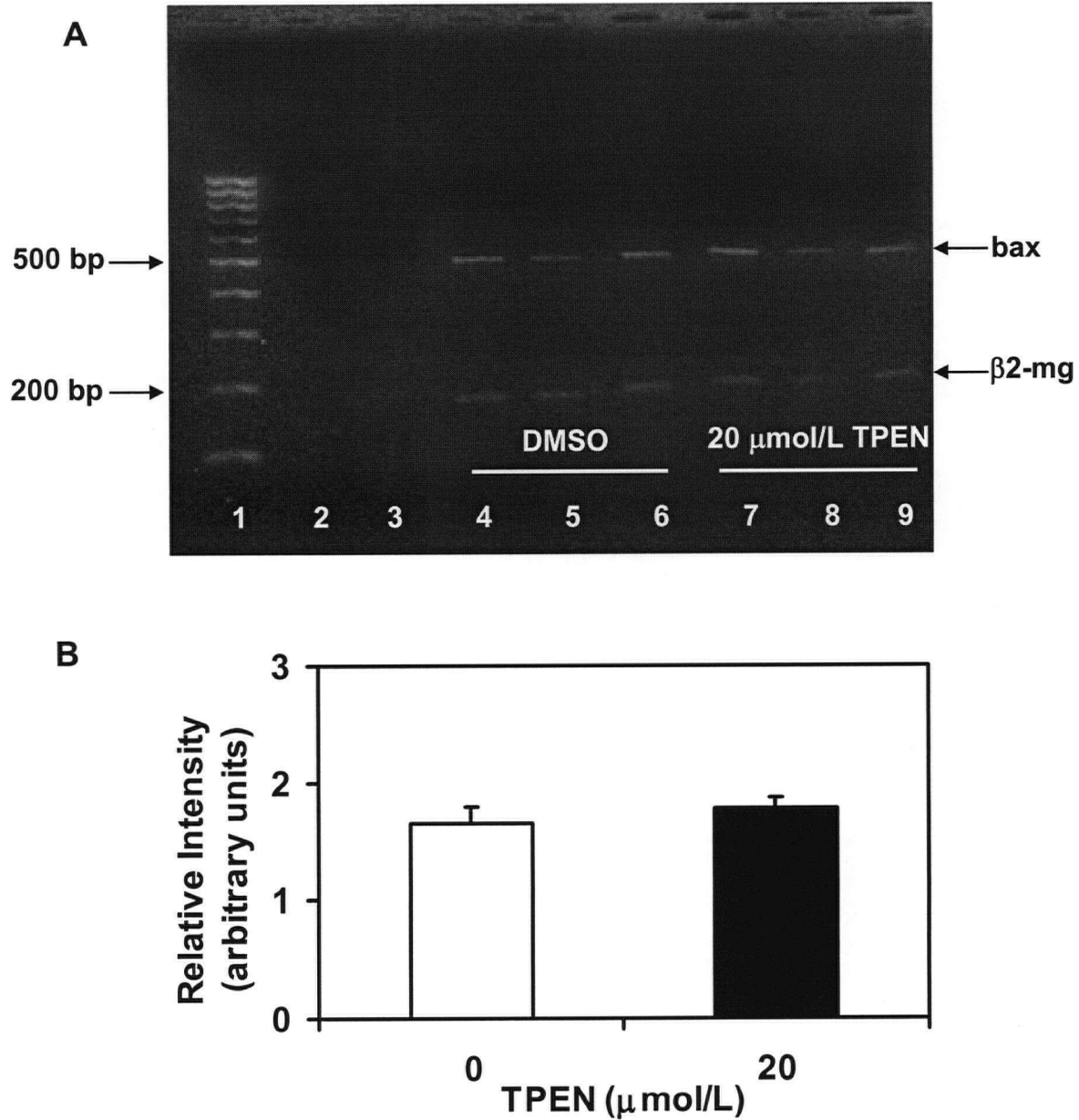
Figure II.8. RT-PCR analysis of Bcl-2 mRNA level in MDA-MB-231 cells treated with TPEN for 8 h. Total RNAs were isolated from cells and were reverse transcribed as described in the Material and Methods. PCR products were subjected to agarose (2%) gel electrophoresis. (A) Representative ethidium bromide-stained agarose gel showing the level of Bcl-2 and co-amplified β2-microglobuin (β2-mg) mRNAs. Lane 1: DNA ladder; Lane 2: negative control (no cDNA); Lane 3: negative control (no primers); Lane 4-6: TPEN 0 μmol/L (DMSO); Lane 7-9: TPEN 20 μmol/L. (B) Relative Bcl-2 mRNA level normalized on the optical density of corresponding co-amplified β2-microglobuin band. Values represent mean $\pm$ SEM (n=5). Means with different letters are significantly different ($p < 0.05$).

73

Figure II.9. RT-PCR analysis of Bax mRNA level in MDA-MB-231 cells treated with TPEN for 8 h. Total RNAs were isolated from cells and were reverse transcribed as described in the Material and Methods. PCR products were subjected to agarose (2%) gel electrophoresis. (A) Representative ethidium bromide-stained agarose gel showing the level of Bax and co-amplified $\beta2$-microglobuin ($\beta2$-mg) mRNAs. Lane 1: DNA ladder; Lane 2: negative control (no cDNA); Lane 3: negative control (no primers); Lane 4-6: TPEN 0 μmol/L (DMSO); Lane 7-9: TPEN 20 μmol/L. (B) Relative Bax mRNA level normalized on the optical density of corresponding co-amplified $\beta2$-microglobuin band. Values represent mean $\pm$ SEM (n=5). Means with different letters are significantly different ($p < 0.05$).

74

Table II-1. PCR primers used for amplification of cDNAs

mRNA		Sequence	Reference
β2-microglobulin	Sense Antisense	5'-GTGGAGCATTCAGACTTGTCTTTCAGC-3' 5'-TTCACTCAATCCAAATGCGGCATCTTC-3'	Gautschi et al., 2001
p53	Sense Antisense	5'-TAGTGTGGTGGTGCCCTATGAGCCG-3' 5'-GTGGGAGGCTGTCAGTGGGGAACAA-3'	Winthrop et al., 1997
gadd45	Sense Antisense	5'-GTGAGTGAGTGCAGAAAGCAGGCG-3' 5'-GAAGTGGATCTGCAGAGCCACATC-3'	Winthrop et al., 1997
bcl-2	Sense Antisense	5'-GGTGCCACCTGTGGTCCACCTG-3' 5'-CTTCACTTGTGGCCCAGATAGG-3'	Zhang et al., 1999
bax	Sense Antisense	5'-CAGCTCTGAGCAGATCATGAAGACA-3' 5'-GCCCATCTTCTTCCAGATGGTGAGC-3'	Zhang et al., 1999

REFERENCES

Allen, R. T., Hunter III, W. J. & Agrawal, D. K. (1997) Morphological and biochemical characterization and analysis of apoptosis. J. Pharm. Toxicol. Methods 37: 215-228.

Barbieri, D., Troiano, L., Grassilli, E., Agnesini, C., Cristofalo, E.A., Monti, D., Capri, M., Cossarizza, A. & Franceschi, C. (1992) Inhibition of apoptosis by zinc: a reappraisal. Biochem. Biophys. Res. Commun. 187: 1256-1261.

Bellamy, C. O. (1997) p53 and apoptosis. Br. Med. Bulletin 53: 522-538.

Bergh, J. (1999) Clinical studies of p53 in treatment and benefit of breast cancer patients. Endocr.-Related Cancer 6: 51-59.

Burns, T. & El-Deiry, W. S. (1999) The p53 pathway and apoptosis. J. Cell. Phyl, 181: 231-239.

Butt, A. J., Firth, S. M., King, M. A. & Baxter, R. C. (2000) Insulin-like growth factor-binding protein-3 modulates expression of bax and bcl-2 and potentiates p53-independent radiation-induced apoptosis in human breast cancer cells. J. Biol. Chem. 275: 38174-39181.

Chai, F., Truong-Tran, A. Q., Ho, L. D. & Zalewski, P. D. (1999) Regulation of caspase activation and apoptosis by cellular zinc fluxes and zinc deprivation: a review. Immu. Cell Biol. 77: 272-278.

Cousins, R. J. (1997) Present Knowledge in Nutrition, 29: 293-306.

Elledge, R. M., Lock-Lim, S., Allred, D. C., Hilsenbeck, S. G. & Cordner, L. (1995) p53 mutation and tamoxifen resistance in breast cancer. Clin. Cancer Res. 1: 1203-1208.

Fanzo, J. C., Reaves, S. K., Cui, L., Zhu, L., Wu, J. Y. J., Wang, Y. R. & Lei, K. Y. (2001) Zinc status affects p53, gadd45 and c-fos expression and caspase-3 activity in human bronchial epithelial cells. Am. J. Physiol Cell Physiol. 281: C751-C757.

Fukamachi, Y., Karasaki, Y., Sugiura, T., Itoh, H., Abe, T., Yamanura, K. & Higashi, K. (1998) Zinc suppresses apoptosis of U937 cells induced by H_2O_2 through an increase of the bcl-2/bax ratio. Biochem. Biophys. Res. Comm. 246: 364-369.

Gautschi, O., Tschopp, S., Olie, R. A., Leech, S. H., Simoes-Wust, A.P., Ziegler, A., Baumann, B., Odermatt, B., Hall, J., Stahel, R. A. & Zangemeister-Wittke, U. (2001). Activity of a novel bcl-2/bcl-xL-bispecific antisense oligonucleotide against tumors of diverse histologic origins. J. Natl. Cancer Inst. 93: 463-471.

Korsmeyer, S. J. (1999) BCL-2 gene family and the regulation of programmed cell death. Cancer Res. 59: 1693-1700.

Lee, R., Woo, W., Wu, B., Kummer, A., Duminy, H. & Xu, Z. (2003) Zinc accumulation in N-Methyl-N-Nitrosourea-induced rat mammary tumors is accompanied by an altered expression of ZnT-1 and metallothionein. Exp. Biol. Med. (Accepted).

Li, Y., Upadhyay, S., Bhuiyan, M. & Sarkar F. H. (1999) Induction of apoptosis in breast cancer cells MDA-MB-231 by genistein. Oncogene 18: 3166-3172.

Lipponen, P. (1999) Apoptosis in breast cancer: relationship with other pathological parameters. Endocr. Relat. Cancer, 6: 13-16.

MacDonald, R. S. (2000) The role of zinc in growth and cell proliferation. J. Nutr. 130: 1500S-1508S.

Martin, S. J., Mazadai, G., Strain, J. J. Cotter, T. G. & Hannigan, B. M. (1991) Programmed cell death (apoptosis) in lymphoid and myeloid cell lines during zinc deficiency. Clin. Exp. Immunol 83: 338-343.

Martin, S. J., Reutelingsperger, C. P. M., McGahon, A. J., Radar, A., van-Schie, R. C., LaFace, D. M. & Green, D. R. (1995) Early distribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpresion of Bcl-2 and Abl. J. Exp. Med. 182: 1545-1556.

McGowan, A. J., Fernandes, R. S., Verhaegen, S. & Cotter, T. G. (1994) Zinc inhibits UV radiation-induced apoptosis but fails to prevent subsequent cell death. Int. J. Radiat. Biol. 66: 343-349.

Nasir, M. S., Fahrni, C. J., Suhy, D. A., Kolodsick, K. J., Singer, C. P. & O'Halloran, T. V. (1999) The chemical cell biology of zinc: structure and intracellular fluorescence of a zinc-quinolinesulfonamide complex. J. Biol. Inorg. Chem. 4: 775-783.

Nodera, M., Yangisawa, H.. & Wada, O. (2001) Increased apoptosis in a variety of tissues of zinc-deficient rats. Life Sci. 69: 1639-1649.

Parat, M. O., Richard, M. J., Meplan, C., Favier, A. & Beani, J. C. (1999) Impairment of cultured cell proliferation and metallothionein expression by metal chlelator NNN'N'-tetrakis-(2-pyridylmethyl)ethylenediamine. Biol. Trace. Elem. Res. 70: 51-68.

Paski, S. C. & Xu, Z. (2001) Labile intracellular zinc is associated with 3T3 cell growth. J. Nutr. Biochem. 12: 655-661.

Pellegrini, M. & Strasser, A. (1999) The portrait of the bcl-2 protein family: life, death and the whole picture. J. Clin. Immuno. 19: 365-377.

Prasad, A. S., Beck, F. W., Endre, L., Hanschu, W., Kukuruga, M. & Kumar, G. (1996) Zinc deficiency affects cell cycle and deoxythymidine kinase gene expression in HUT78 cells. J. Lab. Clin.Med.128: 51-60.

Reaves, S. K., Fanzo, J. C., Arima, F. K., Wu, J. Y. J., Wang, Y. R. & Lei, K. Y. (2000) Expression of the p53 tumor suppressor gene is up-regulated by depletion of intracellular zinc in HepG2 cells. J. Nutr. 130: 1688-1694.

Saikumar, P., Dong, Z., Mikhalilov, V., Denton, M., Weinberg, J.M. & Venkatachalam, M.A. (1999) Apoptosis: definition, mechanisms, and relevance to disease. Am. J. Med. 107: 489-506.

Shumaker, D. K., Vann, L. R., Goldberg, M. W., Allen, T. D. & Wilson, K. L. (1998) TPEN, a Zn^{2+}/Fe^{2+} chelator with low affinity for Ca^{2+}, inhibits lamin assembly, destabilizes nuclear architecture and may independently protect nuclei from apoptosis in vitro. Cell Calcium 23: 151-164.

Smith, M.L., Fornace, A.J. (1996) Mammalian DNA damage-inducible genes associated with growth arrest and apoptosis. Mutation Res./Rev. Genetic Toxicol. 340: 109-124.

Smith, P. J., Wiltshire, M., Davies, S., Chin, S. F., Campbell, A. K. & Errington, R. J. (2002) DNA damage-induced $[Zn2^+]_I$ transients: correlation with cell cycle arrest and apoptosis in lymphoma cells. Am. J. Physiol. Cell Physiol. 283: C609-C622.

Truong-Tran, A. Q., Ho, L. H., Chai, F. & Zalewski, P. D. (2000) Cellular zinc fluxes and the Regulation of Apoptosis/Gene-directed cell death. J. Nutr. 130: 1459S-1466S.

Vallee, B. L. & Falchuk, K. H. (1993) The biochemical basis of zinc physiology. Am. Physiol. Soc., 73: 79-118.

Waring, P., Egan, M., Braithwaite, A.,Mullbacher, N. & Siaarda, A. (1990) Apoptosis induced in macrophages and T blasts by the mycotoxin sporodesmin and protection by Zn salts. Int. J. Pharmacol. 12: 445-457.

Winthrop, M. D., Denardo, S. J., Muenzer, J. T., Chi, S. G. & Gumerlock, P. H. (1997) p53-independent response of a human breast carcinoma xenograft to radioimmunotherapy. Cancer 80: 2529-2537.

Woo, W. & Xu, Z. (2002) Body zinc distribution profile during N-methyl-N-nitrosourea-induced mammary tumorigenesis in rats at various levels of dietary zinc intake. Biol. Trace Elem. Res. 87: 157-169.

Zalewski, P. D., Forbes, I. J. & Betts, W. H. (1993) Correlation of apoptosis with change in intracellular labile zinc using Zinquin. Biochem J., 296: 403-408.

Zhang, G. J., Kimijima, I., Onda, M., Kanno, M., Sato, H., Watanabe, T., Tsuchiya, A., Abe, R. & Takenoshita, S. (1999) Tamoxifen-induced apoptosis in breast cancer cells relates to down-regulation of bcl-2, but not bax and bcl-xL, without alteration of p53 protein levels. Clin.Cancer Res. 5: 2971-2977.

CHAPTER III

GENERAL DISCUSSION AND CONCLUSIONS

1. Cell Line Selection

In exploring the linkage between zinc and breast cancer cell growth, two other human breast carcinoma cell lines (MCF-7 and T47D), and a non-transformed, fibrocystic human breast epithelial cell line (MCF-10) were studied. These cell lines were chosen because of the differences in apoptotic gene profile, such as p53 and Bcl-2 family, involved in the apoptosis pathway (Table AI.1). Since the expression of these genes has been shown to be affected by zinc, these cell lines were used in an attempt to facilitate comparisons to provide some insights on how zinc regulates apoptosis in human breast cancer cells.

2. LIPZ was Important to Cell Growth

2.1. TPEN-Induced LIPZ Depletion was Cell Line-Specific

It is well established that zinc is essential for cell growth and proliferation (MacDonald, 2000). But, information is scarce on the mechanism of zinc homeostasis at the cellular level (Vallee and Falchuk, 1993). Recent identification of the more dynamic subcellular zinc pools, LIPZ, which is readily influenced by zinc depletion or supplementation, demonstrates the complexity of zinc control at the cellular level (Chai et al., 1999). The optimal level of LIPZ required to support cell growth is not established. Based on the three human breast cancer cell lines and human fibrocystic breast cell line studied in this project, the LIPZ sizes appeared to be cell line-specific. It varied from MDA-MB-231 cells (20-38 pmol/10^6 cells; Figure II.1), T47D cells (24-28 pmol/10^6 cells; Figure AII.2), MCF-10 cells (28-38 pmol/10^6 cells; Figure AII.3) to MCF-7 cells (54-61 pmol/10^6 cells;

Figure AII.1). The susceptibility of the cell line to TPEN-induced LIPZ depletion in the decreasing order: MDA-MB-231 > MCF-10 > T47D > MCF-7. However, TPEN treatments did not induce any changes in the total cellular zinc contents in the four cell lines tested. Likewise, total cellular zinc contents, in the nanomole range, also varied from cell line to cell line with the highest zinc content in MDA-MB-231 cells, followed by MCF-7 cells, and T47D cells (Figure II.2; Figure AII.4 and AII.5). Interestingly, although the total cellular zinc content was the lowest in fibrocystic MCF-10 cell line (Figure AII.6), its LIPZ size was higher than that in T47D cells. Nevertheless, the size of LIPZ was independent of total cellular zinc content. As increased cell proliferation and DNA synthesis are associated with an increased LIPZ size (Paski and Xu, 2001), it is possible that the difference in LIPZ size reflected cell line-specific requirement for LIPZ for growth. Moreover, the reduced LIPZ size coupled with lack of changes in total cellular zinc and an association between reduced LIPZ size and decreased cell number and cell viability suggested that an adequate LIPZ size is critical to cell integrity.

2.2. TPEN-Induced LIPZ Depletion was Associated with Reduced Cell Growth and Increased Death

TPEN treatments reduced cell numbers and lowered cell viability in a dose-dependent manner in all three breast cancer cell lines (Figure II.3, and II.4; Figure AII.7, AII.8, AII.10, AII.11 and AII.12). The reductions reached a significant level when cells were treated with higher levels and long incubation of TPEN (e.g. 20 μmol/L; 8 h). A similar reduction was observed in the fibrocystic MCF-10 cells (Figure AII.9 and AII.13). Since cell number counts simply provide the number of cells present and do not differentiate live cells from

81

dead cells, MTT reduction assay was used to assess cell viability. This is a colourimetric assay that is dependent on the mitochondrial metabolic activity of viable cells. The amount of MTT reduced to formazan by the mitochondrial succinate-tetraozolium reductase corresponds to the functional growth of the cells. In this study, treating the cells with TPEN reduced cell viability in all four cell lines, with the reduction being more pronounced at higher TPEN concentrations (e.g.10 or 20 μmol/L) in combination with longer treatment duration (e.g. 4 or 8 h) (Figure II.4; Figure AII.10, AII.11, AII.12 and AII.13). These effects of TPEN on the cell viability were in general agreement with the cell number data, which suggested that reduced cell viability was a contributing factor towards reduced cell numbers. Moreover, loss of viability is usually indicative of cells in non-proliferating/resting stages, and even dying or dead cells. Since depletion of LIPZ has been shown to induce apoptosis in a number of cell lines (Zalewski et al., 1993; Parat, 1999; Truong-Tran et al., 2000), reduced cell number and cell viability reported herein could collectively indicate that increased cell death was a contributing factor in TPEN-induced reduction in cell number, and thus overall cell growth.

To further characterize the possible cause of a TPEN-induced reduction in the overall cell growth, the Annexin V- FLUOS and PI staining assay was used to simultaneously sort the live cells from the dead cells, and the necrotic cells from the apoptotic cells. Cell line-dependent responses were observed as well. TPEN treatment at a higher concentration (20 μmol/L) reduced live cells, increased necrotic and apoptotic cell death (Figure II.5; Figure AII.14, AII.15, and AII.16). In T47D, the same TPEN treatment did not affect the percentage of necrotic cells, but a likewise 12% increase in apoptosis was observed (Figure

AII.15). Moreover, the magnitude of TPEN-induced apoptosis is higher in the three breast cancer cell lines, in comparison to the fibrocystic cell line. Therefore, it appears that the TPEN-induced LIPZ depletion may be more detrimental to the fast-proliferating cancerous cells. However, it is important to note that the analysis of cell deaths using Annexin-V-FLUOS and PI does not have sufficient sophistication to differentiate late apoptotic cells from necrotic cells. This is because at the late stage of apoptosis, the cells that have undergone apoptosis have lost their membrane integrity. As a result, the cells can be labeled with both Annexin-V-FLUOS and PI and counted as necrotic cells. Therefore, it is likely that apoptosis was underestimated while necrosis was overestimated. Nevertheless, the results reported herein are consistent with findings in the literature that the depletion of LIPZ elevated apoptotic cell deaths.

2.3. TPEN-Induced LIPZ Depletion was Associated with Altered Expression of Apoptotic Regulators

Zinc is involved in the complex apoptosis pathway and is considered the physiological regulator of this form of death. Yet, the precise mechanisms involved are largely unclear. Zinc may exert its action on apoptosis through its association with p53 tumour suppressor proteins and Bcl-2 family proteins. In order to elucidate the possible causes of an increased apoptotic cell death resulted from TPEN treatment, the mRNA level of several zinc-related apoptotic regulatory genes was assessed by RT-PCR. Based on the cell growth and death data, MDA-MB-231 and T47D were chosen as the candidate cell lines for further investigation, due to their responsiveness to TPEN treatments. For MDA-MB-231 cells, TPEN treatment (20 μmol/L; 8h) reduced mRNA levels of p53 (Figure II.6), gadd45

(Figure II.7), and bcl-2 (Figure II.8) by 18, 36, and 47%, respectively, while the bax mRNA level was not affected (Figure II.9). The bax : bcl-2 ratio in the TPEN treated cells was 2.2, which was 1.8 times of the ratio (1.2) in the cells cultured in the control medium. For T47D cells, the same TPEN treatment reduced mRNA levels of gadd45 by 35% (Figure AII.18), while p53 (Figure AII.17) and bax (Figure AII.19) mRNA levels were unaffected. Bcl-2 mRNA levels was not detected in this cell line under our experimental conditions. Apoptosis can be both p53-dependent and -independent (Bellamy, 1997) in a species, tissue, and cell type-specific manner. As postulated in the earlier chapter, it appears that LIPZ-depletion-induced apoptosis in MDA-MB-231 cells is p53-independent and is regulated by the proteins in the Bcl-2 family. The mechanism for T47D cells is less apparent due to incomplete expression data, and expression of other apoptosis regulatory proteins needs to be investigated.

3. Cellular Zinc Level was Strictly Regulated

Strict cellular zinc homeostasis regulates the level of zinc uptake and intracellular zinc distribution. Total cellular zinc level is to some extent independent of extracellular zinc concentration in the culture media. In C6 rat glioma cells, the total cellular zinc level remains constant with extracellular zinc concentration up to a threshold of 100 μmol/L (Haase and Beyersmann, 1999). Similarly, findings from our lab also show that total cellular zinc concentration is the same between the cells grown in a low zinc medium (0.2 μmol/L) and the cells grown in an adequate zinc medium (5.2 μmol/L). The intracellular distribution of cellular zinc, with 30-40% in nucleus, 50% in the cytosol, and rest associated with membranes, is controlled by zinc transporters (Vallee and Falchuk, 1993; Gaither and Eide,

2001). Some of these zinc transporters are responsible for sequestration of zinc into zinc-storing vesicles, know as zincosomes, trafficking of cellular zinc, and insertion of zinc into nascent proteins (Suhy and O'Halloran, 1996; Beyersmann and Haase, 2001). Membrane zinc transporters, ZnT-2 and ZnT-3, move zinc from cytosol into vesicles. ZnT-2 is responsible for zinc storage during extremely low levels of zinc in the culture media (Nasir et al., 1999). Therefore, zincosomes may have a role in supplying zinc when zinc level is low the cellular environment.

4. Overall Conclusion and Future Directions

In this thesis project, the relationship between LIPZ and breast cancer cell growth were explored. The results indicated an optimal level of LIPZ was critical to the overall growth of human breast cancer cells, such that the deprivation of LIPZ reduced cell viability and survival, and promoted cell deaths. The expression profile of the apoptotic regulatory protein genes provided some insights into the possible mechanisms involved in LIPZ depletion-induced growth responses. However, apoptosis is a complex process, which can be activated by multiple interweaving pathways. It is also possible that these multiple pathways can work concertedly to arrive at the same death outcome. Translational expressions of the apoptotic regulatory factors need to be determined, as changes at the mRNA level do not necessarily give accurate accounts of the expressions and activities of the protein products. The rapid removal of LIPZ via chelation is by no means physiological, and may not mimic any biological situation. There are still questions about the cellular uptake of TPEN into the cells, and whether TPEN is chelating LIPZ as well as chelating a certain percentage of

extracellular zinc en route to entering the cells. Hence, further studies are needed to ascertain the role of LIPZ in the growth of human breast cancer cells.

5. References

Beyersmann, D. & Haase, H. (2001) Functions of zinc in signaling, proliferation and differentiation of mammalian cells. BioMetals 14: 331-341.

Chai, F., Truong-Tran, A. Q., Ho, L. D. & Zalewski, P. D. (1999) Regulation of caspase activation and apoptosis by cellular zinc fluxes and zinc deprivation: a review. Immunol. Cell Biol. 77: 272-278.

Gaither, L. A. & Eide, D. J. (2001a) Eukaryotic zinc transporter and their regulation. BioMetals 14: 251-270.

Hasse, H. & Beyersmann, D. (1999) Uptake and intracellular distribution of labile and total Zn (II) in C6 rat glioma cells investigated with fluorescent probes and atomic absorption. BioMetals 12: 247-254.

MacDonald, R. S. (2000) The role of zinc in growth and cell proliferation. J. Nutr. 130: 1500S-1508S.

Nasir, M. S., Fahrni, C. J., Suhy, D. A., Kolodsick, K. J., Singer, C. P. & O'Halloran, T. V. (1999) The chemical cell biology of zinc: structure and intracellular fluorescence of a zinc-quinolinesulfonamide complex. J. Biol. Inorg. Chem 4: 775-783.

Parat, M. O., Richard, M. J., Meplan, C., Favier, A. & Beani, J. C. (1999) Impairment of cultured cell proliferation and metallothionein expression by metal chlelator NNN'N'-tetrakis-(2-pyridylmethyl)ethylenediamine. Biol. Trace. Elem. Res. 70: 51-68.

Paski, S. C. & Xu, Z. (2001) Labile intracellular zinc is associated with 3T3 cell growth. J. Nutr. Biochem. 12: 655-661.

Suhy, D. A. & O'Halloran, T. V. (1996) Metal-responsive gene regulation and the zinc metalloregulatory model. Met. Ions Biol. Syst. 32: 557-578.

Truong-Tran, A. Q., Ho, L. H., Chai, F. & Zalewski, P. D. (2000a) Cellular zinc fluxes and the regulation of apoptosis/gene-directed cell death. J. Nutr. 130: 1459s-1466s.

Vallee, B. L. & Falchuk, K. H. (1993) The biochemical basis of zinc physiology. Am. Physiol. Soc. 73: 79-118.

Zalewski, P. D., Forbes, I. J. & Betts, W. H. (1993) Correlation of apoptosis with change in intracellular labile zinc using Zinquin. Biochem. J., 296: 403-408.

APPENDICES

APPENDIX I.

Cell Culture System

This study involved three human breast cancer cell lines (MCF-7, MDA-MB-231 and T47D) and a human fibrocystic breast cell line (MCF-10) as a control. These cell lines were chosen because of their differential expressions of the regulatory proteins involved in the apoptosis pathway (Table AI.1.). The human breast cancer cells (American Type Culture Collection, Manassas, VA, U.S.A.) were maintained in their respective culture media (Table AI-2) in T-75 flasks and incubated at 37°C in 5% CO_2. Zinc concentrations for the culture media (Table I.2.) were measured using a flame atomic absorption spectrophotometer (Atomic Absorption Spectrophotometer, model 2380, Perkin Elmer, Norwalk, CT, U.S.A.).

Table AI.1. Characterization of cell lines

Cell Line	p53	Bcl-2	Bax / Bak
MCF-7	+	+	+
MDA-MB-231	Mutant	+	+
T47D	Mutant	-/+ (?)	+ (?)
MCF-10	+	-/+	-/+ (?)

Table AI.2. Culture information on cell lines

Cell Line	Culture Medium	[Zn] μmol/L
MCF-7	DMEM +10% FBS	4.9
MDA-MB-231	DMEM + 10% FBS	4.8
T47D	RPMI 1640 + 10% FBS	4.0
MCF-10	DMEM/F12 +5% HS	2.6

Effects of TPEN on the Growth of Human Breast Cancer Cells

The cells were cultured in 96-well microplates or flasks with known initial seeding densities. They were 10^4 cells/well (96-well microplate), 10^5 cells/T-25 flask and 3×10^6 cells/T-75 flask. The cells were cultured in their respective media until reaching exponential growth (72 h). After the initial growth period, cells were subjected to various treatments of TPEN (0, 5, 10, or 20 μmol/L) and incubation durations (0, 1, 2, 4, or 8 h).

1. Effects of TPEN treatment on the LIPZ
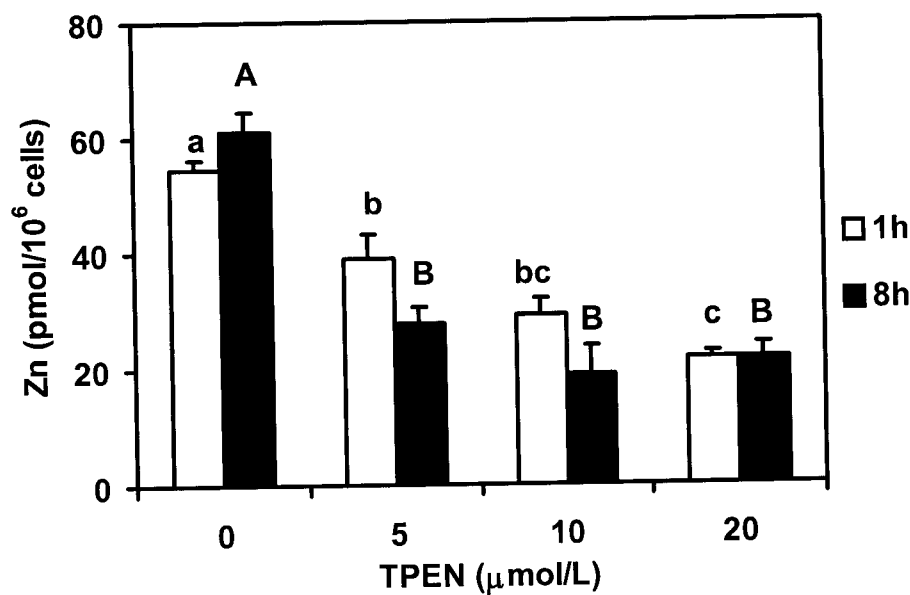
Figure AII.1. Effect of TPEN concentration and treatment duration on the LIPZ content in MCF-7 human breast cancer cells. Cells were treated with TPEN (0 (DMSO), 5, 10, or 20 μmol/L) for 1h (open bars) or 8 h (filled bars). Values represent mean ± SEM (n = 4). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).
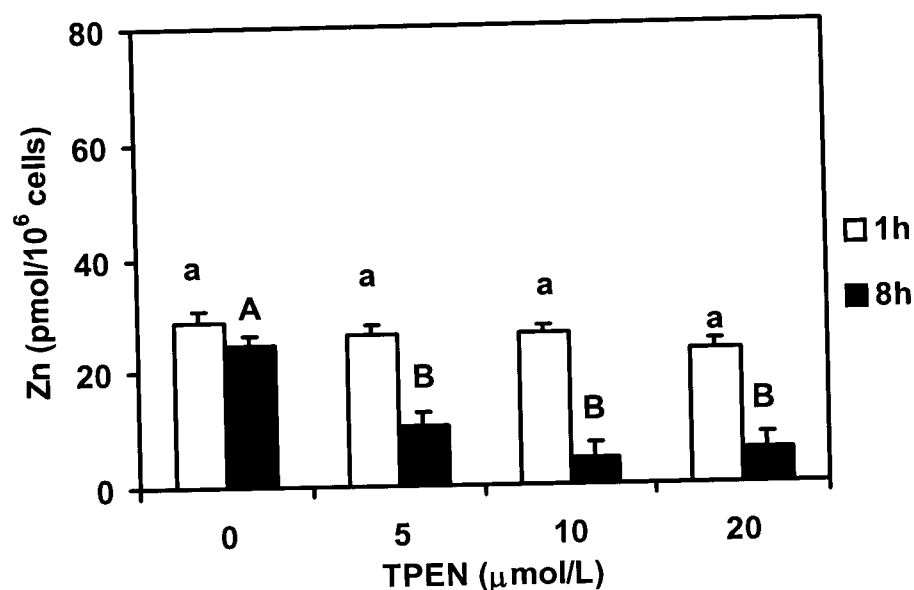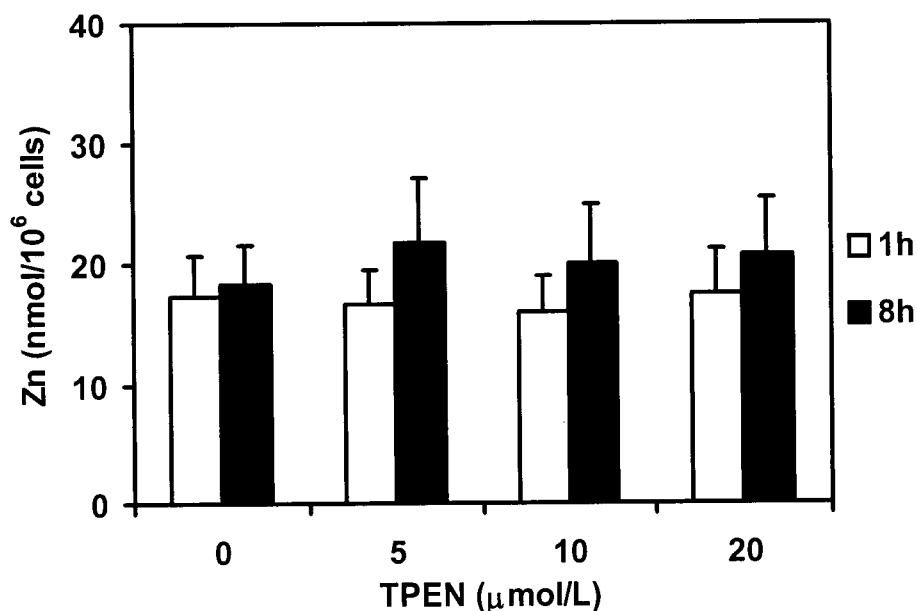
Figure AII.2. Effect of TPEN concentration and treatment duration on the LIPZ content in T47D human breast cancer cells. Cells were treated with TPEN (0 (DMSO), 5, 10, or 20 μmol/L) for 1h (open bars) or 8h (filled bars). Values represent mean ± SEM (n = 4). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).
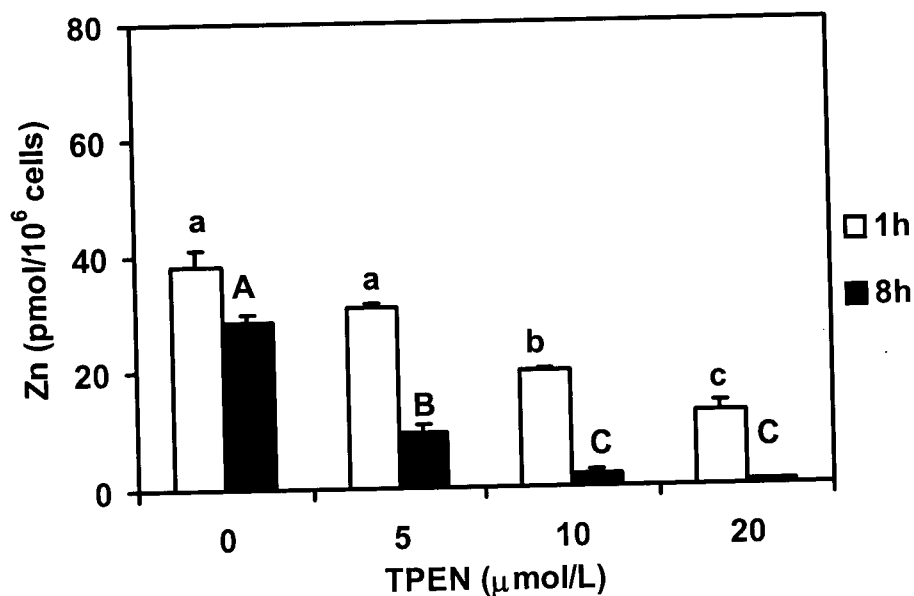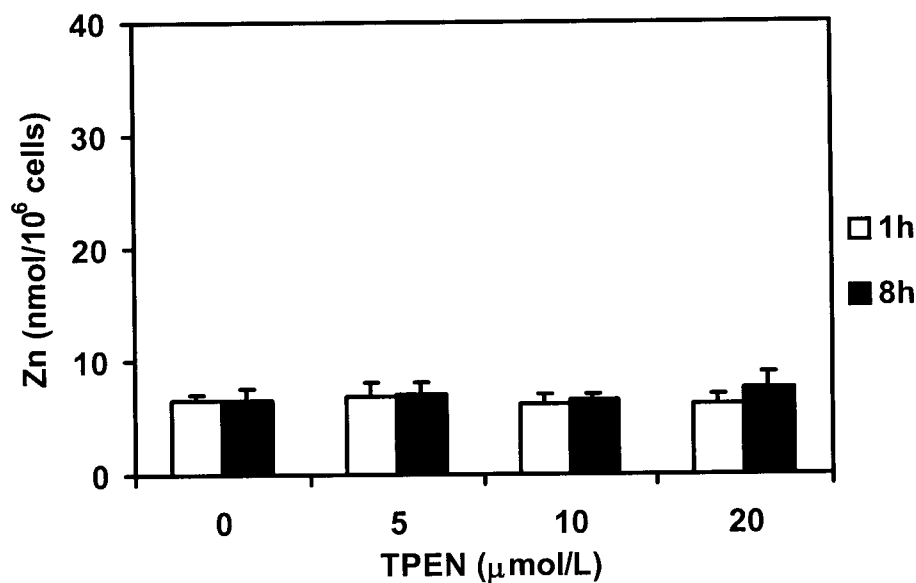

Figure AII.3. Effect of TPEN concentration and treatment duration on the LIPZ content in MCF-10 human fibrocystic breast cells. Cells were treated with TPEN (0 (DMSO), 5, 10, or 20 μmol/L) for 1h (open bars) or 8h (filled bars). Values represent mean ± SEM (n = 4). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).

2. Effects of TPEN treatment on total cellular zinc
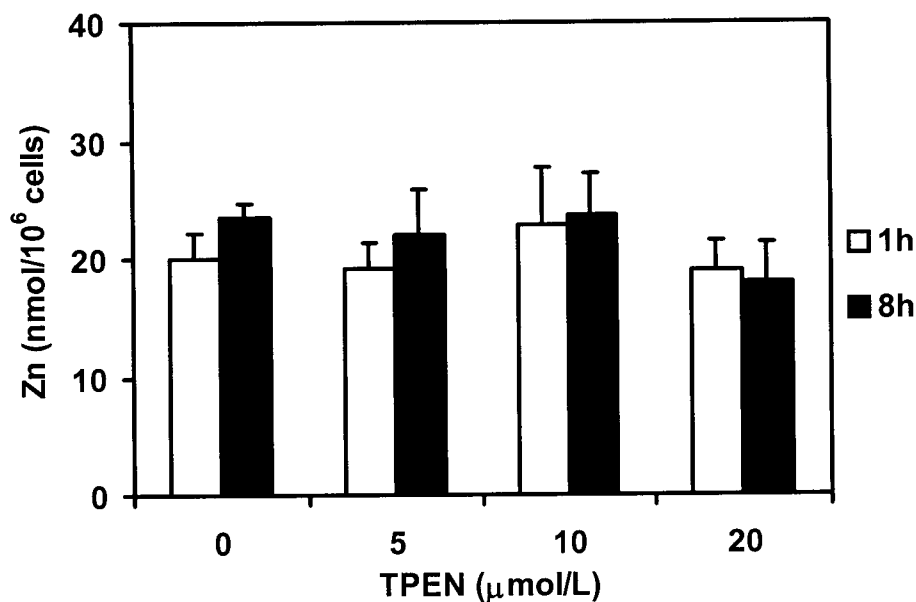
Figure AII.4. Effect of TPEN concentration and treatment duration on the total cellular zinc content in MCF-7 human breast cancer cells. Cells were treated with TPEN (0 (DMSO), 5, 10, or 20 μmol/L) for 1 h (open bars) or 8 h (filled bars). Values represent mean ± SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).
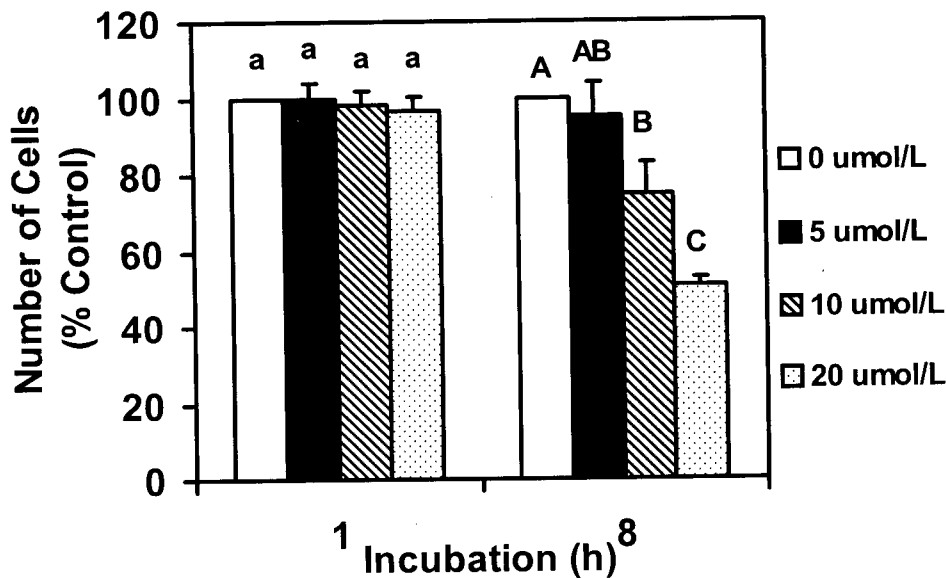

Figure AII.5. Effect of TPEN concentration and treatment duration on the total cellular zinc content in T47D human breast cancer cells. Cells were treated with TPEN (0 (DMSO), 5, 10, or 20 μmol/L) for 1 h (open bars) or 8 h (filled bars). Values represent mean ± SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).

Figure AII.6. Effect of TPEN concentration and treatment duration on the total cellular zinc content in MCF-10 human fibrocystic breast cells. Cells were treated with TPEN (0 (DMSO), 5, 10, or 20 μmol/L) for 1 h (open bars) or 8 h (filled bars). Values represent mean ± SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).
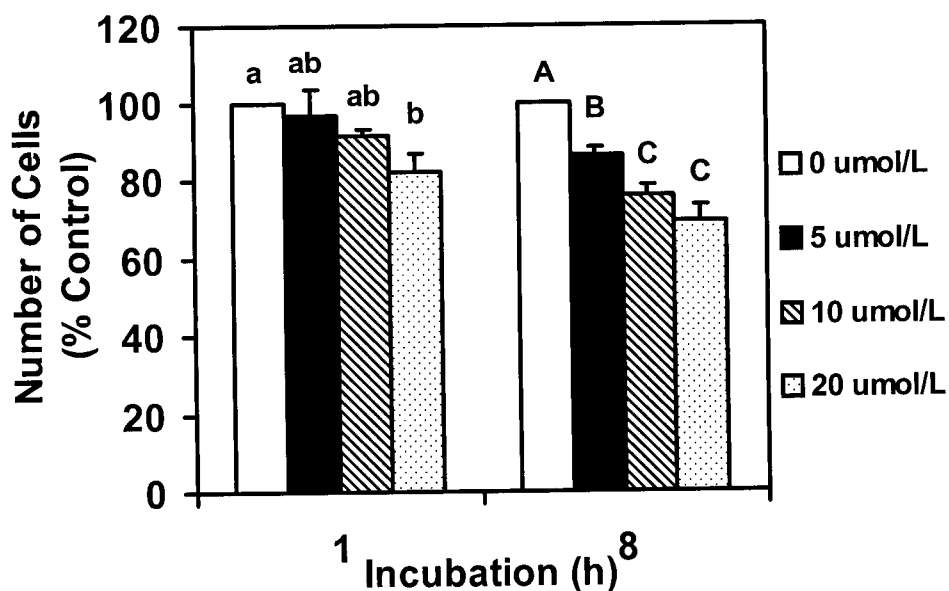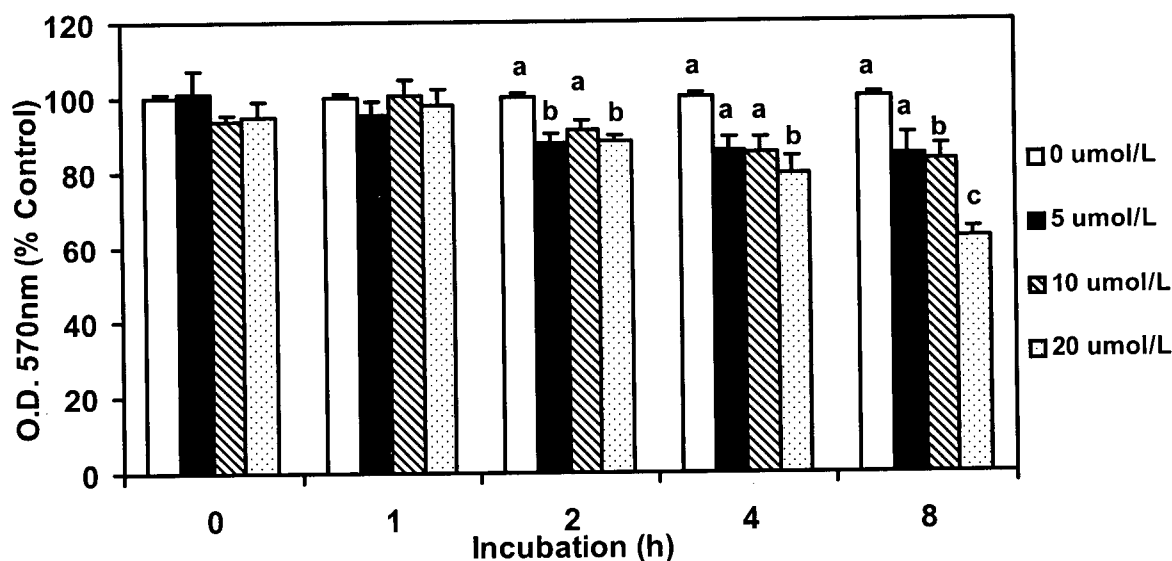
3. Effects of TPEN treatment on overall cell growth
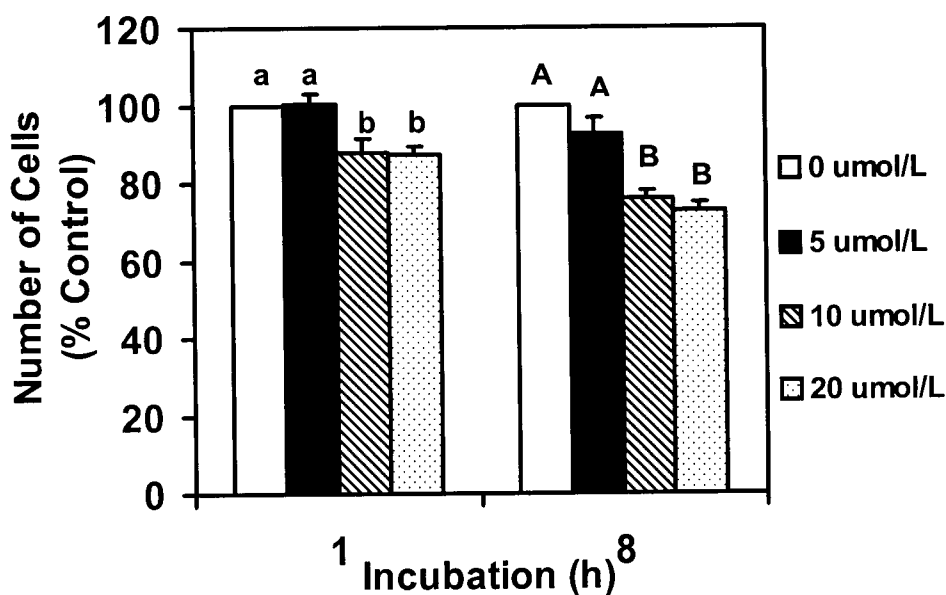
Figure AII.7. Effect of TPEN concentration and treatment duration on overall cell growth in MCF-7 human breast cancer cells. Cells were treated with TPEN (0 (DMSO) (open bars), 5 (filled bars), 10 (striped bars), or 20 (dotted bars) μmol/L) for 1 or 8 h. Values represent mean ± SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).

Figure AII.8. Effect of TPEN concentration and treatment duration on overall cell growth in T47D human breast cancer cells. Cells were treated with TPEN (0 (DMSO) (open bars), 5 (filled bars), 10 (striped bars), or 20 (dotted bars) μmol/L) for 1 or 8 h. Values represent mean ± SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).
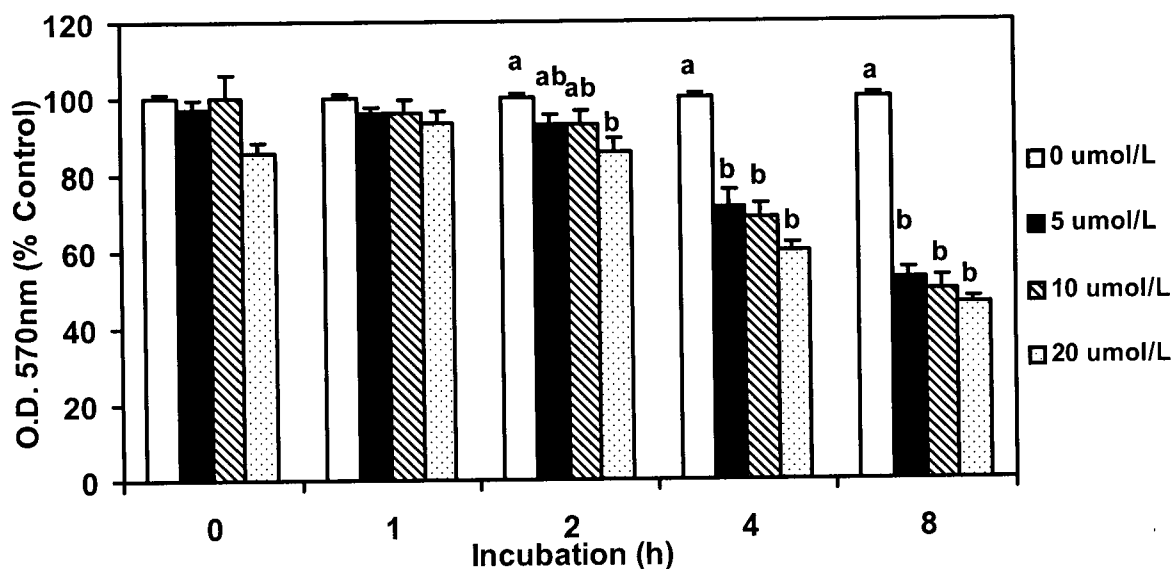

Figure AII.9. Effect of TPEN concentration and treatment duration on overall cell growth in MCF-10 human fibrocystic breast cells. Cells were treated with TPEN (0 (DMSO) (open bars), 5 (filled bars), 10 (striped bars), or 20 (dotted bars) μmol/L) for 1 or 8 h. Values represent mean ± SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).

94

4. Effects of TPEN treatment on cell viability

Figure AII.10. Effect of TPEN concentration and treatment duration on cell viability in MCF-7 human breast cancer cells. Cells were treated with TPEN (0 (DMSO) (open bars), 5 (filled bars), 10 (striped bars), or 20 (dotted bars) $\mu mol/L$) for 0, 1, 2, 4 or 8 h. Values represent mean $\pm$ SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).
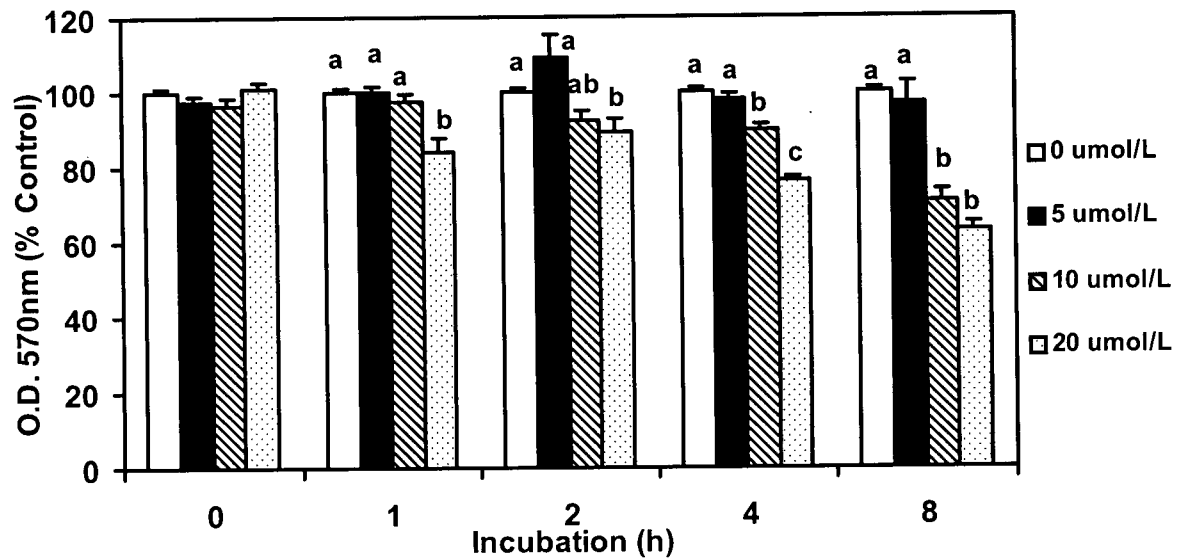

Figure AII.11. Effect of TPEN concentration and treatment duration on cell viability in MDA-MB-231 human breast cancer cells. Cells were treated with TPEN (0 (DMSO) (open bars), 5 (filled bars), 10 (striped bars), or 20 (dotted bars) $\mu mol/L$) for 0, 1, 2, 4 or 8 h. Values represent mean $\pm$ SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).
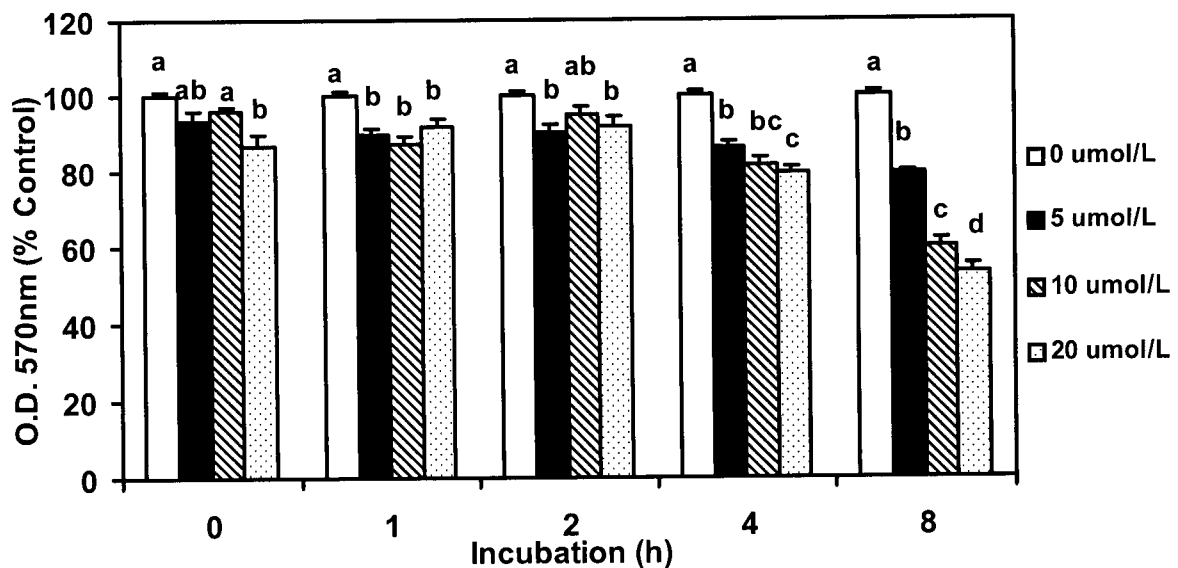
Figure AII.12. Effect of TPEN concentration and treatment duration on cell viability in T47D human breast cancer cells. Cells were treated with TPEN (0 (DMSO) (open bars), 5 (filled bars), 10 (striped bars), or 20 (dotted bars) μmol/L) for 0, 1, 2, 4 or 8 h. Values represent mean ± SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).

Figure AII.13. Effect of TPEN concentration and treatment duration on cell viability in MCF-10 human fibrocystic breast cells. Cells were treated with TPEN (0 (DMSO) (open bars), 5 (filled bars), 10 (striped bars), or 20 (dotted bars) μmol/L) for 0, 1, 2, 4 or 8 h. Values represent mean ± SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).

96

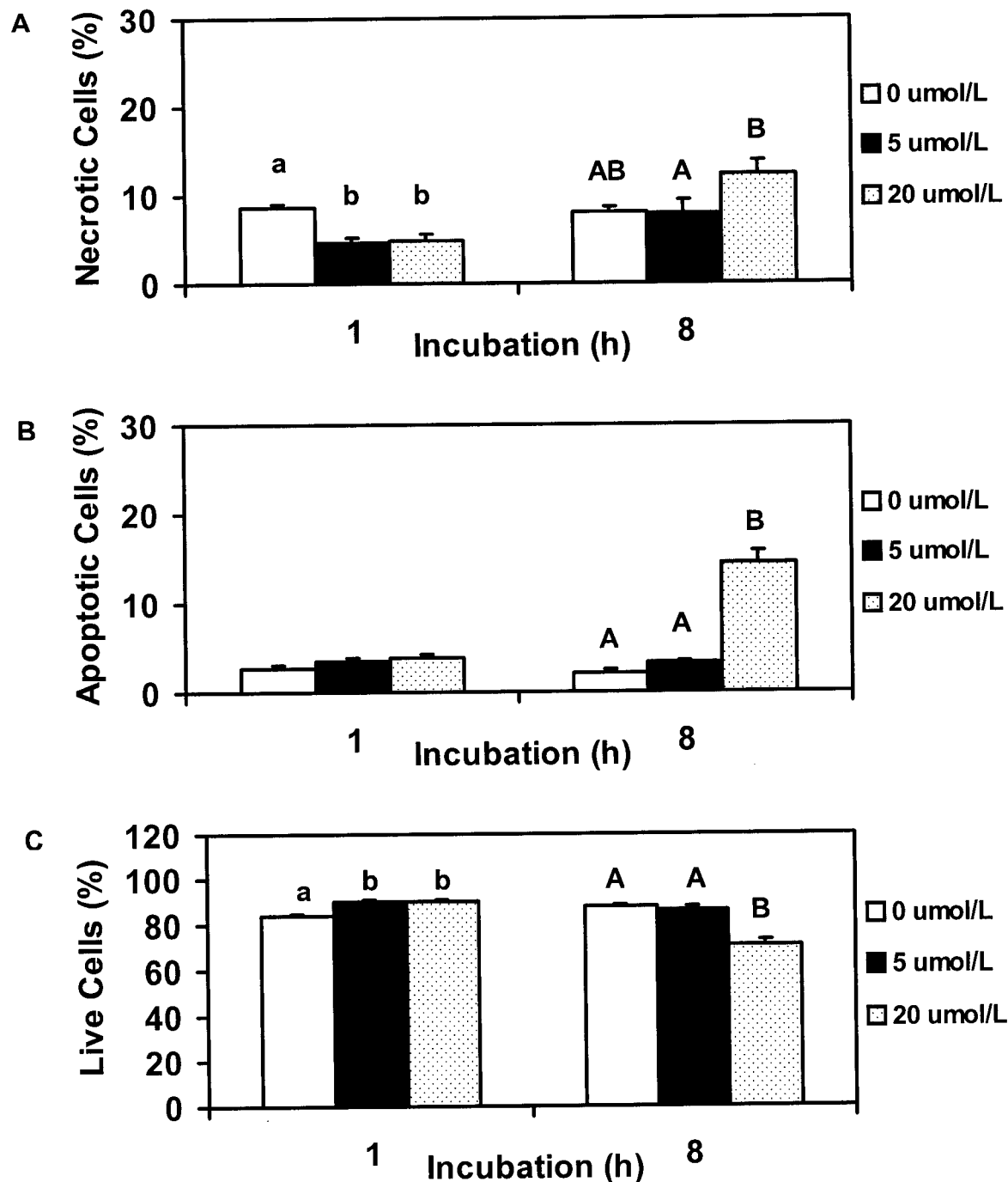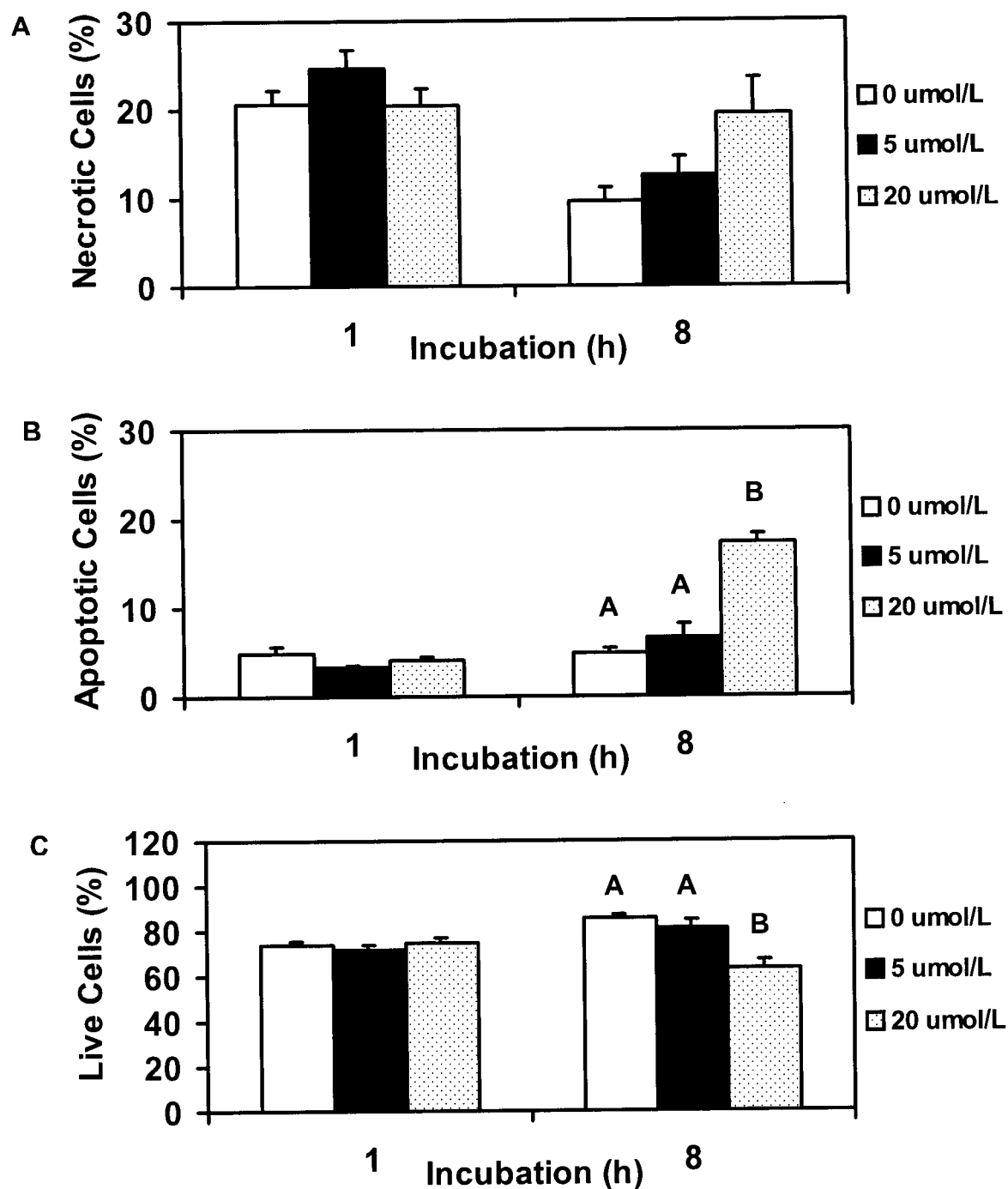## 5. Effects of TPEN treatment on cell survival and deaths

Figure AII.14. Effect of TPEN concentration and treatment duration on cell survival and deaths in MCF-7 human breast cancer cells. (A) Necrotic cells. (B) Apoptotic cells. (C) Live cells. Cells were treated with TPEN (0 (DMSO) (open bars), 5 (filled bars), or 20 (dotted bars) μmol/L) for 1 or 8 h. Values represent mean ± SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).

Figure AII.15. Effect of TPEN concentration and treatment duration on cell survival and deaths in T47D human breast cancer cells. (A) Necrotic cells. (B) Apoptotic cells. (C) Live cells. Cells were treated with TPEN (0 (DMSO) (open bars), 5 (filled bars), or 20 (dotted bars) μmol/L) for 1 or 8 h. Values represent mean ± SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).
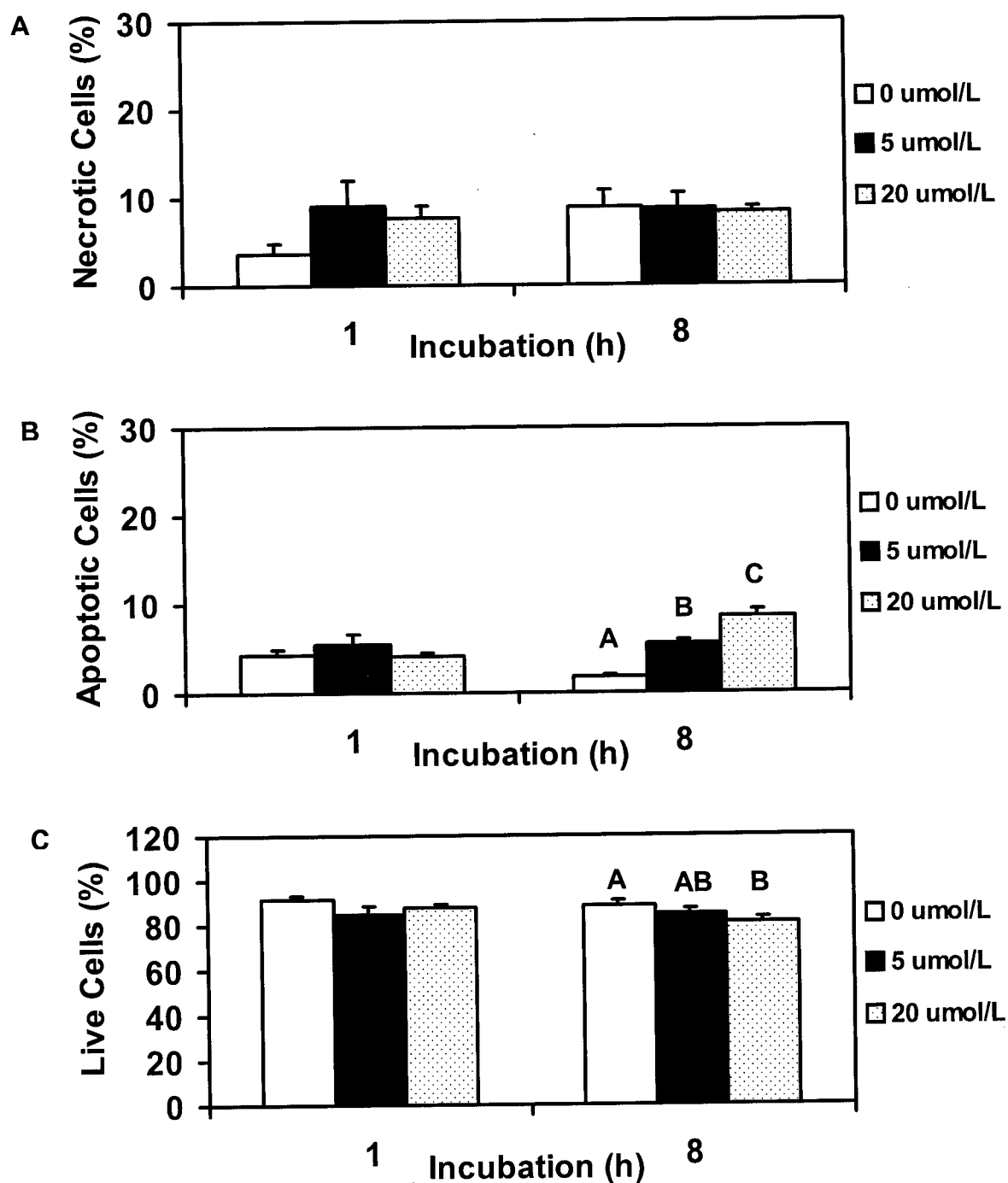
Figure AII.16. Effect of TPEN concentration and treatment duration on cell survival and deaths in MCF-10 human fibrocystic breast cells. (A) Necrotic cells. (B) Apoptotic cells. (C) Live cells. Cells were treated with TPEN (0 (DMSO) (open bars), 5 (filled bars), or 20 (dotted bars) μmol/L) for 1 or 8 h. Values represent mean ± SEM (n = 6). The means among different TPEN concentrations within the same treatment duration with different letters are significantly different ($p < 0.05$).

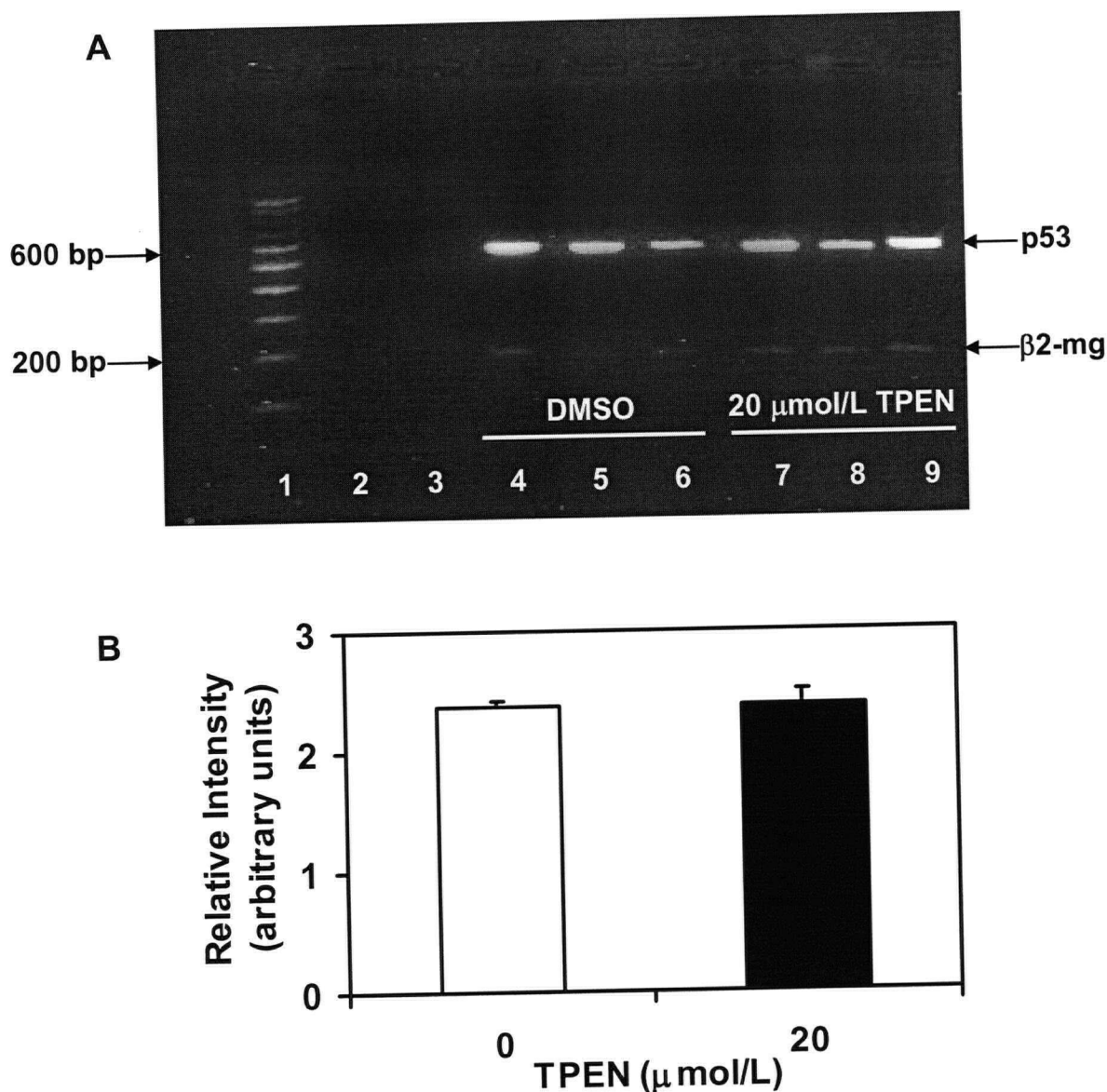## 6. Effects of TPEN treatment on mRNA levels of apoptotic-regulatory genes

Figure AII.17. RT-PCR analysis of p53 mRNA level in T47D human breast cancer cells treated with TPEN for 8 h. Total RNAs were isolated from cells and were reverse transcribed as described in the Material and Methods in Chapter II. PCR products were subjected to agarose (2 %) gel electrophoresis. (A) Ethidium bromide-stained agarose gel showing representative p53 mRNA levels and (β2-mg) levels. Lane 1: DNA ladder; Lane 2: negative control (no cDNA); Lane 3: negative control (no primers); Lane 4-6: TPEN 0 μmol/L (DMSO) 8 h; Lane 7-9: TPEN 20 μmol/L 8 h. (B) Relative p53 mRNA level normalized on the optical density of the corresponding co-amplified β2-microglobuin band. Values represent mean ± SEM (n = 5). Means with different letters are significantly different ($p < 0.05$).
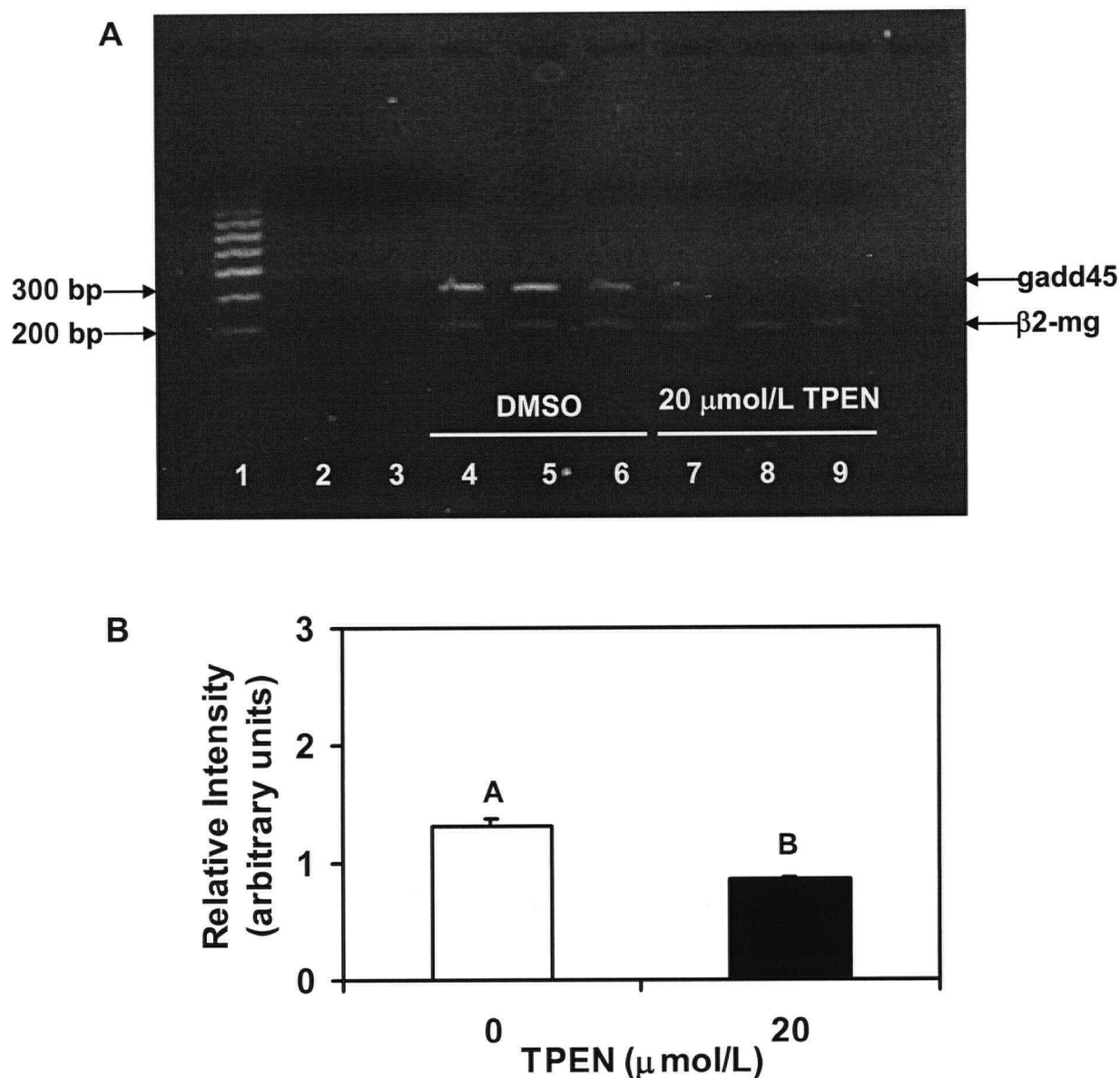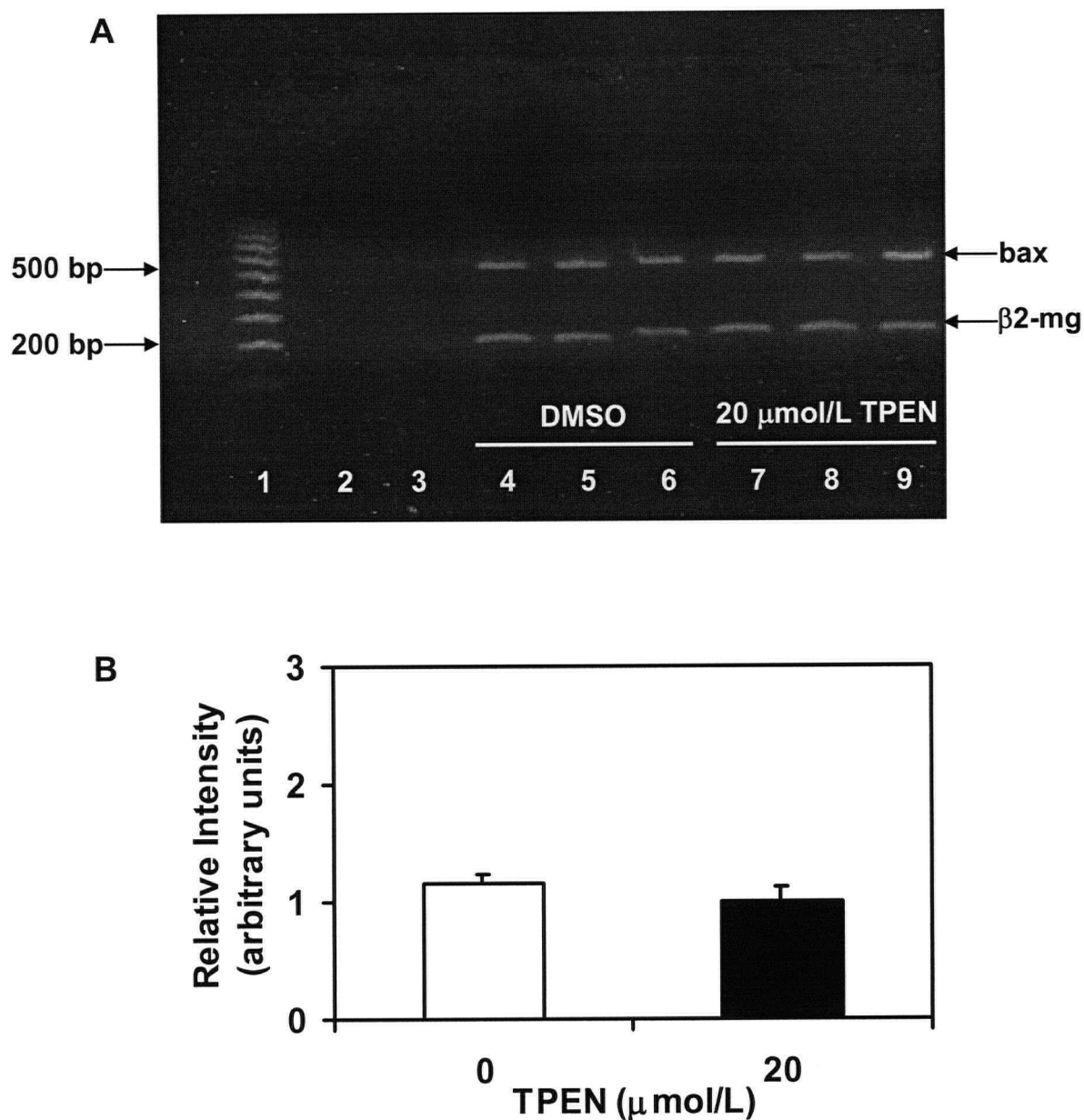
Figure AII-18. RT-PCR analysis of gadd45 mRNA level in T47D human breast cancer cells treated with TPEN for 8 h. Total RNAs were isolated from cells and were reverse transcribed as described in Material and Methods in Chapter II. PCR products were subjected to agarose (2 %) gel electrophoresis. (A) Ethidium bromide-stained agarose gel showing representative gadd45 mRNA levels and co-amplified β2-microglobuin (β2-mg) levels. Lane 1: DNA ladder; Lane 2: negative control (no cDNA); Lane 3: negative control (no primers); Lane 4-6: TPEN 0 μmol/L (DMSO) 8 h; Lane 7-9: TPEN 20 μmol/L 8 h. (B) Relative gadd45 mRNA level normalized on the optical density of the corresponding co-amplified β2-microglobuin band. Values represent mean $\pm$ SEM (n = 5). Means with different letters are significantly different ($p < 0.05$).

Figure AII-19. RT-PCR analysis of bax mRNA level in T47D human breast cancer cells treated with TPEN for 8 h. Total RNAs were isolated from cells and were reverse transcribed as described in Material and Methods in Chapter II. PCR products were subjected to agarose (2 %) gel electrophoresis. (A) Ethidium bromide-stained agarose gel showing representative bax mRNA levels and co-amplified β2-microglobuin (β2-mg) levels. Lane 1: DNA ladder; Lane 2: negative control (no cDNA); Lane 3: negative control (no primers); Lane 4-6: TPEN 0 μmol/L (DMSO) 8 h; Lane 7-9: TPEN 20 μmol/L 8 h. (B) Relative bax mRNA level normalized on the optical density of the corresponding co-amplified β2-microglobuin band. Values represent mean $\pm$ SEM (n = 5). Means with different letters are significantly different ($p < 0.05$).

102