ABSTRACT

Title: MODULATION OF HUMAN TUMOR SUPPRESSOR GENES, GADD45, p53 AND p38 MAPK BY ZINC STATUS IN NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS

Sheung-Mei Shih, Doctor of Philosophy, 2007

Directed By: Professor David K.Y. Lei
Department of Nutrition and Food Science

The effect of zinc status on the cell signaling transduction of tumor suppressor genes, Growth Arrest and DNA Damage inducible gene (Gadd45), p53, and p38 Mitogen Activated Protein Kinase (MAPK) were examined in Normal Human Bronchial Epithelial (NHBE) cells. Cells were cultured for one passage in different concentrations of zinc: < 0.4 μM (ZD, zinc-deficient); 4 μM (ZN, zinc normal) as normal zinc level found in most culture medium; 16 μM (ZA, zinc adequate) represented normal human plasma zinc level; and 32 μM (ZS, zinc supplementation) represented the optimal plasma zinc attainable by oral supplementation. Cell growth inhibition, up-regulation of Gadd45, p53 and p38 MAPK mRNA and protein expressions, and blockage of G2/M cell cycle progression were observed in ZS cells. The siRNA-mediated knocking down of Gadd45 was found to alleviate G2/M blockage partially in ZS cells, which indicated that the blockage is partially Gadd45 dependent. In ZS cells, the enhanced phosphorylation of p38 MAPK and p53 were abrogated after suppressing Gadd45 by siRNA, implicating

that the enhanced phosphorylation of p53 and p38 MAPK were Gadd45 dependent. By using p53 transactivation inhibitor Pifithrin, the upregulated Gadd45 protein, the enhanced Gadd45 promoter activity, and the reduced level of CDK1/Cyclin B1 complex were all restored back to normal levels in ZS cell, implying that these ZS induced changes were p53 dependent. Furthermore, the ZS induced upregulation of Gadd45 expression, displacement of CDC25B from nucleus to cytoplasm, reduction of CDK1/Cyclin B1 complex level, enhancement of the activation and phosphorylation of p53, and delay of G2/M cell cycle progression were normalized by p38 MAPK dominant negative and protein inhibitor SB202190. Thus, the ZS induced changes were dependent on the activation of p38 MAPK. Our data support the involvement of a positive Gadd45, p53 and p38 MAPK feedback loop in response to stress induced by zinc supplementation. These findings demonstrate the importance of p38 MAPK and p53 in the regulation of G2/M cell cycle progression in response to the stress induced by high zinc via Gadd45 and cell cycle checkpoint regulatory proteins, including CDK1, Cyclin B1 and CDC25B.

MODULATION OF HUMAN TUMOR SUPPRESSOR GENES,GADD45, p53 AND p38 MAPK BY ZINC STATUS IN NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS

By

Sheung-Mei Shih

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2007

Advisory Committee:

Professor David K.Y. Lei, Chair
Professor Mickey Parish
Professor Yang Tao
Professor Liangli Yu
Professor Thomas T.Y. Wang

Dedication

For *Mom, Dad*
&
Hon-Kit

Acknowledgements

I would like to thank Dr. David K.Y. Lei, both for his guidance and advice on my research project, and his critical review of my dissertation. He has walked me through all the stages of writing this dissertation. I also would like to express my gratitude to Drs. Parish, Tao, Yu and Wang for their review of my dissertation and serving on my dissertation committee. I am particularly grateful to Dr. Parish for his warm smile and support whenever I need his help. I would like to thank Dr. Norberta Schoene in USDA for her assistance in cell cycle analysis. The help from Ms. Noel Bryden in Dr. Richard Anderson's laboratory in USDA is also highly appreciated. I also want to extend my appreciation to general office's colleagues, especially Mary Pandian, Shirley Pressley, Vera McCoy and Brian Boner, for their warm smiles throughout these whole three years.

My deepest thanks would like to go to my beloved parents, brother and sister for their love and encouragement, without them, I would not be who I am today.

My most heartfelt thanks are due to my loving husband, Hon-Kit who gave me tremendous support throughout the whole graduate studies, M.Phil and Ph.D. I am deeply grateful to his love, support, and consistent encouragement. He teaches me the ways to appreciate molecular biology in life science. Without him, this present dissertation would not be possible. Thank him for bring a loving and harmonious home and make the life in US easier and enjoyable. He is the greatest gift from God.

Table of Contents

Figures and illustrations

Figures and illustrations – *Continued*

Figures and illustrations – *Continued*

Figures and illustrations – *Continued*

Figures and illustrations – *Continued*

CHAPTER I:

INTRODUCTION

In view of the prevalence and clinical significance of zinc deficiency in human populations, as well as extensive use of zinc supplementation in animal production and to a lesser extend in human populations, the present studies were designed to examine the activation of p38, p53 and Gadd45 signaling transduction cascade on G2/M cell cycle progression in Normal Human Bronchial Epithelial (NHBE) cells. NHBE cells are selected because they represent target cells in human lung disease (Stoner et al., 1980), they are more representative of the cell population during lung transformation and they are considered to be progenitor cells for human bronchial cancer. Thus, they can be considered as an excellent model for investigating human lung epithelial cell carcinogenesis (Lechner and LaVeck, 1985).

The Growth Arrest and DNA Damage-inducible gene, Gadd45, has been implicated in the control of cell cycle checkpoint, DNA repair process and signaling transduction. The induction of Gadd45 is dependent on the transactivation of p53, a tumor suppressor gene known for preserving genomic integrity. Upon DNA damage Mitogen-Activated Protein Kinase (MAPK) p38 mediates a variety of cellular behaviors and has been implicated in G2/M cell cycle progression. Various studies demonstrated a functional association between stress-activated MAPK pathway and Gadd45 in response to environmental stresses.

In the current project, we proposed five hypotheses. Firstly, in zinc-supplemented (ZS) NHBE cells, the up-regulated Gadd45 mRNA and protein expressions block G2/M cell cycle progression and inhibit cell growth. Secondly, in ZS NHBE cells, the

upregulated Gadd45 expression is mediated through the p53 dependent pathway. Thirdly, the upregulation of p53 is responsible for the G2/M blockage in ZS NHBE cells induced by the reduced CDK1 and Cyclin B1 complex level. Fourthly, an enhanced p38 MAPK activity induces the displacement of CDC25B from nucleus to cytoplasm compartment as well as a reduction of CDK1/Cyclin B1 complex level, which are responsible for the G2/M blockage in ZS NHBE cells. Fifthly, in ZS NHBE cells, the induction of Gadd45, p38 MAPK, and p53 proteins, which form a positive feedback loop, is resulted from the stress induced by zinc supplementation.

Six main objectives of the present studies were used to test the above hypotheses. Firstly, to measure the Gadd45, p53, p38 mRNA abundances in ZD, ZN, ZA and ZS; Secondly, to quantify protein levels of Gadd45, p53 and p38 MAPK in NHBE cells cultured under different zinc concentrations; Thirdly, to study the influence of Gadd45 siRNA on the expression level of active form of p38 MAPK (phospho-p38) and p53 (phospho-p53); Fourthly, to investigate the influence of knocking down Gadd45 expression by RNA interference on G2/M progression in NHBE cells; Fifthly, to examine the effect of suppressing p53 transactivation activity by Pifithrin on the transcriptional and translational expression of Gadd45, the level of CDK1/Cyclin B1 complex, and G2/M progression in NHBE cells. Sixthly, to establish the influence of suppressing p38 MAPK by dominant negative and inhibitor SB202190 on the expression of Gadd45, phosphorylation of p53, cellular levels of CDK1/Cyclin B1 complex, distribution of CDC25B in nucleus and cytoplasm compartments, and on G2/M progression in NHBE cells.

Experiments were designed using different approaches to uncover the underlining mechanisms responsible for the reduction in cell growth and delay in G2/M cell cycle progression in ZS NHBE cells. Cells were cultured for one passage in different concentrations of zinc: <0.4 μM (ZD) as severe zinc deficient; 4 μM as normal zinc level in culture medium; 16 μM (ZA) as normal human plasma zinc level; and 32 μM (ZS) as the high end of plasma zinc level attainable by oral supplementation. The cell cycle progression was examined by Flow Cytometry. The expressions of p38 MAPK, p53 and Gadd45 mRNA abundance in zinc status were measured by RNase Protection Assay (RPA). The protein levels of p38 MAPK, p53, Gadd45, phospho-p53 (ser15), phospho-p38 MAPK were analyzed by Western Blot. Suppression of Gadd45 was accomplished by the usage of small interference RNA (siRNA) targeting Gadd45 mRNA. The effect of siRNA suppression on Gadd45 was examined by measurement of Gadd45 protein, cell cycle progression, phospho-p53 protein, and phospho-p38 MAPK. The dependency of Gadd45 and p53 expression and G2/M progression on the activation of p38 MAPK was examined not only by administration of p38 MAPK (SB202190) protein inhibitor, but also by applying p38 MAPK dominant negatives, to suppress the expression of p38 MAPK. p38 MAPK kinase activity was measured by non-radioactive kinase assay. The protein levels of CDK1 and Cyclin B1 complex were measured by immunoprecipitation. Gadd45 protein level, cell cycle progression, and the levels of CDK1 and Cyclin B1 complex, were measured after suppressing p38 MAPK. Furthermore, the induction of Gadd45 in the p53-dependent pathway was investigated by suppressing p53 transactivation activity with protein inhibitor Pifithrin.

A better understanding of the mechanisms involved in the protection of normal cells from zinc cytotoxicity is essential for the identification of potential targets, such as Gadd45, p53 and p38 MAPK, and may contribute to the development of future therapeutic approaches for the treatment of human lung diseases.

CHAPTER II:

Literature Review

2.1. Zinc

Introduction

Minerals are inorganic substances that exist naturally in the earth. Many of them are necessary for good health, and are termed as essential nutrients. Minerals are found in bone, tissue, blood, and nerve cells, and are divided into two groups, namely macrominerals and microminerals. Macrominerals are necessary for human health which is needed in relatively large more than 100mg/day. Macrominearals includes sodium, calcium, potassium, chloride, phosphorus, magnesium and sulphur. Microminerals are needed in small amounts of less than 100mg/day. Microminerals include iron, zinc, copper, selenium, manganese, molybdenum, chromium, bromine, fluorine and cobalt.

Zinc belongs to group II B of the transition elements of the periodic table. Its atomic number is 30 and atomic weight is 65.39. It was firstly isolated in 1746 by the chemist S. Margraaf and was named by the Swiss alchemist Theophrastus Bombastus von Hohenheim. A divalent ion under physiological conditions, zinc (~3 g) is the second most abundant trace element in the body after Fe (4 g) and is considerably more abundant than Cu (0.1 g; Solomons, 1988). Zinc is present in all organs, tissues, and body fluids with the highest levels in muscles, liver, kidneys, bones and prostate. Approximately 90% of total body zinc is found in skeletal muscle and bone. Over 95% of total body zinc is bound to proteins within cells and cell membranes. Most of the zinc (75% to 88%) in blood is found in the red blood cell zinc metalloenzyme carbonic anhydrase. In the

plasma, approximately 18% of zinc is bound to alpha-2-macroglobulin, 80% to albumin and 2% to such proteins as transferrin and ceruloplasmin.

Zinc is vital play a role in physiological processes involves in reproduction, development, dark vision adaptation, olfactory activity, insulin homeostasis, and immune defenses. Zinc deficiency can result in stunted growth, immune malfunction, increase infection susceptibility, hypogonadism, anorexia, diarrhea, weight loss, skin changes, oligospermia, delayed wound healing, neural tube defects of the fetus, alopecia, mental lethargy and increased risk for abortion.

Zinc is an essential element in human and animal nutrition with a wide range of biological roles. Zinc plays catalytic, structural or regulatory roles in the more than 300 zinc metalloenzymes that have been identified in biological processes. These enzymes are involved in nucleic acid, protein metabolism and production of energy. Zinc plays a structural role in zinc finger motif. Zinc fingers are used by transcription factors for interacting with DNA and regulation of genes. Another structural role of zinc is in the maintenance of the integrity of biological membranes resulting in their protection against oxidative injury.

Zinc is essential for good health. The recommended daily allowance (RDA) of zinc is 8 mg/day for women and 11 mg/day for men. Daily essential intake is dependent on food, and the daily requirement depends on gender, age, and general status. Women in pregnancy and in lactation have a higher zinc requirement therefore the RDA of zinc increases for lactated women to 12 mg/day (Walsh et al., 1990; Simon-Hettich et al., 2001; Prasad et al., 1995).

In developed countries, zinc deficiency Moderate to severe zinc deficiency is rare.

But people are at risk for mild zinc deficiency. However, it is highly prevalent in developing countries. Several diseases and situations including the autosomal recessive disease acrodermatitis enteropathica, alcoholism, malabsorption, thermal burns, total parenteral nutrition without zinc supplementation and certain drugs, such as diuretics, penicillamine, sodium valproate and ethambutol, predispose human to the development of zinc deficiency. In many of the elderly, zinc intake may be suboptimal, and if compounded with certain drugs and diseases, can lead to mild or even moderate zinc deficiency.

Zinc is an essential dietary factor that mediates a wide range of physiological processes (Vallee & Falchuk, 1993). The influence of zinc on the respiratory system is relatively unknown. Zinc has numerous properties which enable it to modulate the function in airway epithelium and the cells that interact with this tissue which includes: fibroblasts that mediate production of collagen and other extracellular matrix proteins (Sacco et al., 2004); immunoregulatory cells (T and B lymphocytes, macrophages, and antigen-presenting dendritic cells (Stumbles, 1999); and inflammatory cells such as eosinophils, neutrophils, and mast cells (Cohn et al., 2004).

The human body does not store zinc and a constant dietary intake is necessary. Zinc can be obtained from red meat and animal proteins which have a high content of zinc that is bound to ligands capable of facilitating zinc absorption (Solomons, 1988). Other sources of zinc are seafood, dairy foods, cereals, and nuts (Dreosti, 1993). Most vegetables are not good sources of zinc due to the presence of phytate which chelates zinc and inhibits its absorption (Arsenault & Brown, 2003). Diets which are low in animal protein and rich in phytate contribute to the high incidence of zinc deficiency in

many developing countries. Another cause of human primary zinc deficiency can be low zinc content of soils on which cows are raised (Lambein et al., 1994). Inadequate intake of zinc is accompanied by a number of clinical manifestations. In addition, excess of zinc interferes with macromolecular synthesis and function and is cytotoxic.

Zinc balance is maintained through regulated intestinal uptake, fecal excretion, and renal reabsorption (Hambidge & Krebs, 2001). The bulk of body zinc is tightly bound within cellular metalloenzymes and zinc finger proteins (Vallee & Falchuk, 1993). This fixed pool of zinc turns over very slowly and is mainly responsible for housekeeping functions in cellular metabolism and gene expression. The remaining labile zinc is around 10–15% which comprises of more dynamic pools that are readily depleted in Zinc deficiency. While fixed Zinc is distributed uniformly throughout the body, and labile zinc is concentrated in certain tissues and within specific regions within tissues. Labile zinc is usually sequestered in membrane-enclosed organelles including vesicles, granules and Golgi apparatus because labile zinc is potentially toxic to many sulphydryl-dependent cytoplasmic enzymes (Palmiter et al., 1996a). Abundant levels of labile zinc are predominantly seen in cells and tissues involved in secretion, including the prostatic epithelium (secretion of seminal zinc), pancreatic islets (secretion of insulin and glucagon), certain presynaptic neurons (secretion of glutamate), and mast cells (secretion of histamine; Frederickson, 1989; Zalewski et al., 1994b; Ho et al., 2004).

Homeostasis of zinc

Zinc homeostasis is maintained in the gastrointestinal system by the process of absorption of exogenous zinc as well as gastrointestinal secretion and excretion of endogenous zinc (Adamoli et al., 2001; Krebs et al., 2000).

The primary site of absorption of exogenous zinc in humans is the distal duodenum and jejunum. Absorption studies in animal models indicate an inverse relationship between proximal percentage absorption and dietary intakes (Adamoli et al., 2001; Krebs et al., 2000). Free zinc forms complexes with ligands, such as amino acids, phosphates, and other organic acids. These complexes are more easily absorbed than zinc ions (Adamoli et al., 2001; Krebs et al., 2000).

Inositol hexaphosphates and pentaphosphates (phytic acid) bind zinc and form poorly soluble complexes that results in reduced absorption of zinc. Phytate is present in staple foods includes cereals, corn, and rice, which exerts a highly negative effect on zinc absorption. Iron and cadmium can also have an adverse effect on zinc absorption (Krebs et al., 2000; Lonnerdal et al., 2000).

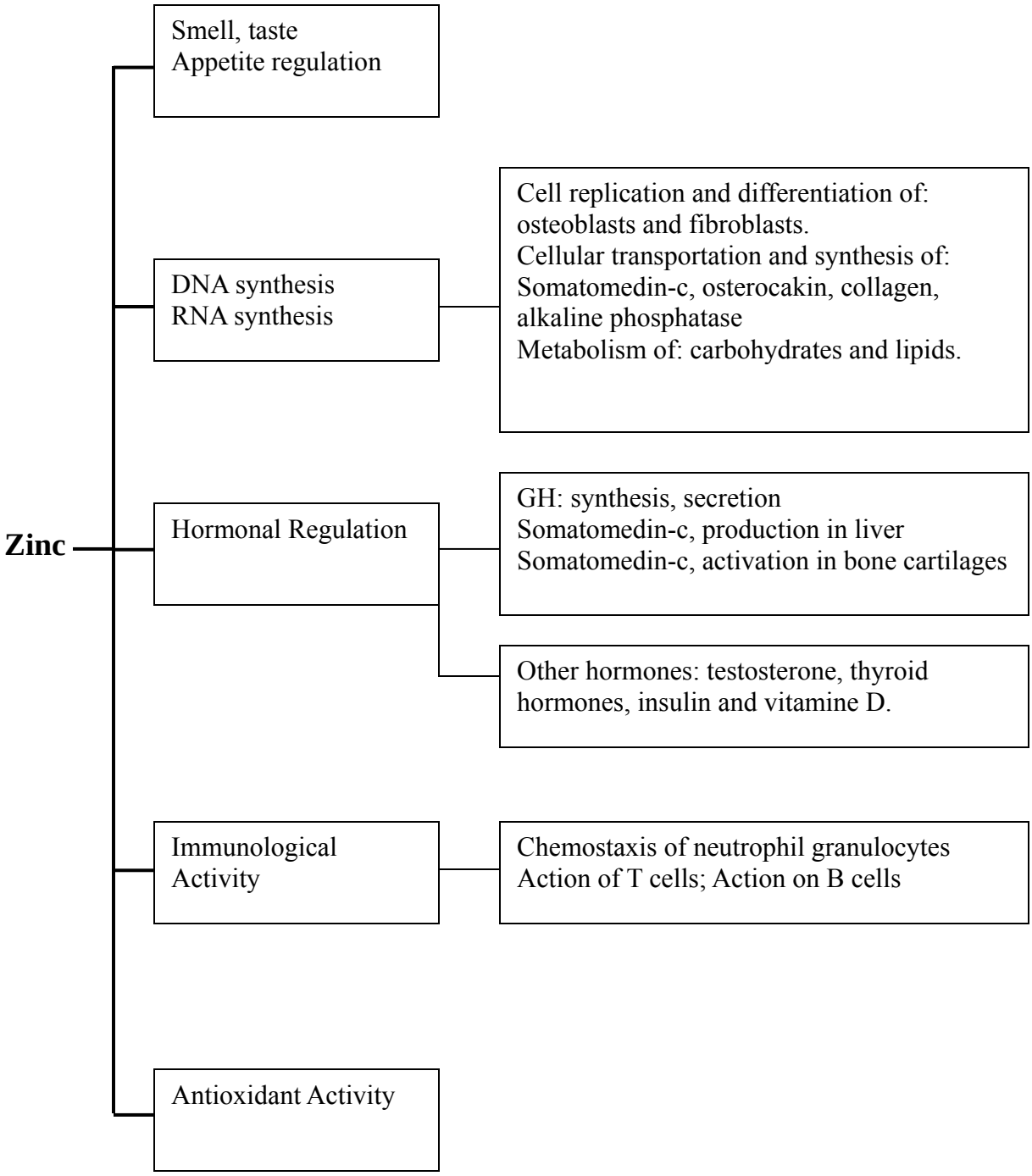
Although the amount of protein in a meal produces a positive effect on zinc absorption, individual proteins may act differently. For instances, casein has a modest inhibitory effect on zinc absorption compared with other proteins sources. Amino acids, such as histidine and methionine, and low molecular weight complexing agents, such as EDTA and organic acid (citrate), are known to exert a positive effect on zinc absorption, and they have been used in zinc supplementation (Krebs et al., 2000; Lonnerdal et al., 2000; Sandstrom et al., 1989).

Table 1. Zinc concentration in foods

Food Groups: Average zinc content (mg/kg)	
Meat	52
Offal	52
Nuts	30
Meat products	25
Poultry	15
Eggs	13
Milk products	12
Bread	9.8
Cereals	9.9
Fish	8.0
Sugars and preserves	5.5
Canned vegetables	4.2
Green vegetables	3.9
Milk	3.9
Potatoes	3.3
Other vegetables	2.4
Fresh fruit	0.85
Fruit products	0.63
Oils and fats	0.5
Beverages	0.14

Source: Ysart et al., 2000.

Fig. 1.1. Functional roles of zinc



Source: Salgueiro et al., 2002.

2.2. Airway Epithelium

Introduction

Airway epithelium is the physical barrier that separates the airway connective tissue and smooth muscle from the airway lumenal contents. The smooth muscle may contain a number of potentially noxious substances such as allergens, dust, and gaseous pollutants. In the absence of an intact epithelial barrier, these potential hazardous substances can stimulate smooth muscle contraction and initiate an inflammatory reaction in the underlying submucosa (Folkerts & Nijkamp, 1998; Thompson, 1998; Holgate et al., 1999). Airway epithelium comprises up to 8 different cell types: ciliated columnar cells (the predominant cell type), secretary cells (goblet cells, serous cells, clara cells, small mucous granule cells, brush cells, and neuroendocrine cells) and basal cells (Takizawa, 1990). The basal cells serve to anchor columnar cells to the basement membrane, and as precursors of columnar and secretary airway epithelium cells (Ayers & Jeffery, 1988). To provide energy for ciliary beating, the apical cytoplasm contains abundant mitochondria (Mills et al., 1999). Other airway epithelial cells secrete high molecular weight mucopolysaccharides (mucin) into the periciliary or epithelial lining fluid (ELF). Foreign particles trapped in the mucin are cleared by the beating of the cilia in a process known as mucociliary clearance (Rogers, 2004). Amongst other factors secreted into ELF are anti-bacterial agents such as lysozyme, collectins, and β-defensins (Cole et al., 1999; Van de Wetering et al., 2004), and anti-oxidants such as glutathione and ascorbic acid (Deaton et al., 2004). Therefore, the airway epithelium plays a critical role in maintaining sterile, undamaged airway tracts through clearance of irritants and other noxious particles. Apart from these protective functions, the airway epithelium regulates airway physiology

through production of smooth muscle relaxant factors such as prostaglandin E2 and nitric oxide and enzymes which catabolize smooth muscle contractile agonists (Holgate et al., 1999). Airway epithelium is susceptible to damage by inhaled pollutants, proteases released from inflammatory cells and from dust mites (Asokananthan et al., 2002), and by reactive oxygen species (ROS), which may arise from endogenous sources include mitochondria, as well as from exogenous sources e.g., inflammatory cells and cigarette smoke (Truong-Tran et al., 2001a). Susceptibility of airway epithelium to damage may, in part be genetically determined, since only a proportion of smokers appear to be susceptible to the airway damage that results in physiological decline in airway function (Fletcher & Peto, 1977). The role of abnormal repair mechanisms in the response to cigarette smoke is yet to be determined. Damage to airway epithelium is a significant factor also in the pathogenesis of a number of chronic inflammatory airway diseases including asthma, chronic obstructive pulmonary disease, and cystic fibrosis. As a consequence of repeated damage and repair, the epithelium becomes remodeled and over-produces mucin, growth factors, and pro-inflammatory cytokines such as TNFa, which act to further aggravate the inflammation and resulting airway hyper-responsiveness (Woolcock & Barnes, 1992). Apoptosis, a mechanism of cell death whereby the cell fragments by a controlled, energy-dependent process into apoptotic bodies, is important for cell turnover in epithelial tissues, including airway epithelium (Rosenblatt et al., 2001; Kerr, 2002). Airway epithelium cells are susceptible to apoptosis by various stimuli including corticosteroids, pro-inflammatory cytokines, and ROS (Wen et al., 1997; Dorscheid et al., 2001; Bucchieri et al., 2002). Apoptotic airway epithelium cells undergo morphological and biochemical changes which facilitate their rapid

clearance by phagocytosis or by shedding into the lumen.. These dying cells are removed by mucociliary transport and are either swallowed or coughed up. Important effector proteins driving apoptosis are the caspase family of enzymes (especially caspases-3 and -6) which exist in healthy cells as inactive precursors (pro-caspases) and become active in cells about to apoptosis (Salvesen & Abrams, 2004). This regional distribution of procaspase-3 may have important implications for spatio-temporal aspects of caspase activation and apoptosis in these cells, since the initial damage leading to apoptosis may be more likely to occur at the luminal surface from contact with inhaled pollutants, allergens, and oxidants. In addition, there may be high local concentrations of ROS released from apical mitochondria as a by-product of energy formation for ciliary beating. The localization of procaspase-3 to the apical cytoplasm could therefore provide an early response mechanism to luminal toxins, allowing the airway epithelium cells to die in a controlled manner by apoptosis. While apoptosis of damaged airway epithelium cells is likely to be beneficial in the normal respiratory tract, excessive and inappropriate apoptosis of airway epithelium cells may contribute to the pathogenesis of chronic disease.

2.3. Zinc in airway epithelium cells

Localization of zinc

Studies using fluorescent dye Zinquin have shown that airway epithelium is rich in labile zinc. Zinquin fluorescence was most intense at the luminal surface of the airway epithelium in cryostat sections of sheep, pig, and mouse trachea and lung (Truong-Tran et al., 2000; Truong-Tran et al., 2002). The distribution of zinc was investigated after obtaining single cell suspensions of ciliated columnar airway epithelial cells prepared by tracheobronchial or nasal brushing. In more than 95% of these cells (sheep or human), Zinquin fluorescence was most intense in the apical cytoplasm (Carter et al., 2002). In approximately 60% of the cells, there was a prominent, diffuse apical fluorescence that may largely be due to zinc in cytoplasmic vesicles, some perinuclear and others are in proximity to the apical membrane.

Zinc turnover in airway epithelium

There is likely to be a considerable flux of zinc into and across airway epithelium. Firstly, there is zinc uptake at the basolateral surface from zinc -containing plasma albumin in sub-epithelial capillaries. From studies in other tissues, it is likely that the hZIP family of transporters is involved in the plasma membrane uptake, and the zinc is packaged in cytoplasmic vesicles which migrate to the perinuclear region and apical cytoplasm. Studies have shown strong apical immunolocalization of zinc transporter 4 (ZnT4) in ciliated human bronchial and nasal epithelial cells, as well as weaker localization to the basolateral plasma membrane. ZnT4 has not previously been found to mediate cellular zinc uptake in other types of cell. Rather since ZnT4 is largely localized

to the membranes of cytoplasmic vesicles (Murgia et al., 1999; Ranaldi et al., 2002), it may play a primary role in vesicular uptake of zinc and its translocation to the apical cytoplasm. Here zinc may be released from vesicles and incorporated into the microtubules of basal bodies (Truong-Tran et al., 2000). Other zinc may be disposed around the apical mitochondria or interact with cytoplasmic metalloproteins in this region such as procaspase-3 (zinc -regulated protein) and Cu/Zn superoxide dismutase (Cu/Zn SOD) (Carter et al., 2002). At least some of the zinc may be destined for secretion into epithelial lining fluid (ELF). Measurements of the zinc content of this fluid should be informative. While most airway epithelium zinc likely derives from sub-epithelial capillaries, we cannot exclude that some zinc enters at the mucosal surface of the lumen by reabsorption of secreted zinc. Certainly, polarized epithelial cells can internalize macromolecules across either the apical or basolateral membranes (Apodaca, 2001).

Functional role of zinc in Airway epithelium

Zinc has potential cytoprotective, secretory, growth-promoting, and signaling functions not only for airway epithelium but also for the cells which interact with this tissue.

Signal transduction of zinc

The concept that zinc ions may participate, like calcium ions, as second messengers in cell signaling was first proposed two decades ago (Williams, 1984), however, evidence for supporting this concept is only now beginning to emerge. Zinc ions have now been shown to dynamically regulate three important signaling pathways, stimulating the ras

tyrosine kinase and phosphatidylinositol 3-kinase/Akt pathways and suppressing protein kinase C (Bruinsma et al., 2002; Hajnal, 2002; Korichneva et al., 2002; Wu et al., 2003). For example, Wu et al. (2003) demonstrated that zinc supplementation of primary human airway epithelium in vitro and rat airway epithelium in vivo resulted in proteasome-mediated degradation and loss of function of PTEN, a tumor suppressor protein which dephosphorylates PIP3 and negatively regulates the phosphatidylinositol 3-kinase/Akt pathway, thereby stimulating cell growth and suppressing apoptosis in airway epithelium. Two further studies are of particular relevance to airway smooth muscle contraction. Firstly, zinc concentration as low as 1 μM was found to bridge two trans-membrane domains of the beta2 adrenoreceptor, resulting in a positive allosteric modulation of agonist binding (Swaminath et al., 2003). This finding is interesting for two reasons. Agonists of beta 2 adrenoreceptor relax airway smooth muscle and are widely used bronchodilators in the treatment of asthma. Moreover, at a pharmacological level, there may be benefit in adding zinc with bronchodilators. At a physiological level, a fall in zinc levels in airways below a certain threshold may enhance broncho-constriction through an imbalance in sympathetic cholinergic systems. Furthermore, zinc may influence bronchoconstriction.

Antioxidant activity of zinc

Airway epithelium is vulnerable to a range of oxidants derived internally or externally from atmospheric oxidants and from inflammatory cells during acute or chronic inflammation of the airways (Wright et al., 1994). To combat these oxidants, airway epithelium and its secretions contain a variety of anti-oxidants including catalase,

vitamins C and E, glutathione, and two forms of superoxide dismutase (Wright et al., 1994). Using protein magnetic resonance imaging to monitor hyperoxia-induced damage in rats, studies showed that in vivo zinc deficiency results in increased oxidative stress in the lung (Taylor et al. 1997). When zinc was repleted in the diet, damage from hyperoxia exposure was prevented. Some of the anti-oxidant effects of zinc are due to stabilization of sulphydryls and membrane lipids (Powell, 2000; Carter et al., 2002) and suppression of nitric oxide production (Cui et al., 1999). Zinc is also a component of a major anti-oxidant enzyme in airway epithelium, Cu/Zn SOD. Larsen et al. (2000) demonstrated the importance of Cu/Zn SOD by showing that transgenic mice with elevated levels of this enzyme in the lungs were more resistant to allergen-induced hyper-responsiveness than wild-type mice. Using immunofluorescence in human bronchial and sheep tracheal airway epithelial cells, studies have shown that Cu/Zn SOD is localized to the same apical region as labile zinc (Carter et al., 2002). The zinc in this metalloenzyme appears to be readily exchangeable (Estevez et al., 1999) and may be reactive with Zinquin. Although the role of zinc in Cu/Zn SOD is unclear, its removal is known to promote catalysis of peroxynitrite-mediated tyrosine nitration, resulting in protein oxidation and consequent cellular damage (Estevez et al., 1999).

Zinc stabilizes the cytoskeleton and cell membranes of airway epithelium

Zinc deficiency alters the lipid composition, enzyme activity, and protein composition of the plasma membrane and associated cytoskeleton thereby increasing membrane permeability (Hennig et al. 1999). Studies are needed to determine whether zinc deficiency leads to an increase in airway epithelium permeability and whether

altered airway epithelium zinc levels are a factor in increased permeability of inflamed airways. The prominent labeling of the ciliary apparatus by Zinquin may indicate another role for zinc in the formation, integrity, or beating of cilia. Tubulin is known to bind zinc (Serrano et al., 1988) and contains sulphydryls that are susceptible to oxidation (Roychowdhury et al., 2000). Since there is evidence that both ciliary beat frequency and integrity of cilia are reduced by cigarette smoke and other oxidants (Pettersson et al., 1985; Sisson et al., 1994), which may affect one or more of these sulphydryls may be critical.

Effects of zinc supplementation in airway epithelium

There have been no detailed studies of zinc levels in airway inflammatory disorders. However, there has been some interest in the relationship between zinc deficiency and cystic fibrosis, an airway disease that, like asthma, is characterized by hypersecretion of mucin into the airway lumen. The first indications that infants with cystic fibrosis may have abnormalities in zinc homeostasis emerged from analysis of plasma zinc concentrations from the Colorado Newborn Screening Program. This survey suggested that zinc status was frequently suboptimal by 6 weeks of age in infants with cystic fibrosis (Krebs et al., 2000). Nearly 30% had plasma zinc concentrations below the normal range and therapy using pancreatic enzyme replacement resulted in a normalization of plasma zinc. A conclusion from these studies was that plasma zinc concentrations should be evaluated in infants or children with cystic fibrosis, especially those who have poor growth, and that zinc should be included among the specific micronutrients in the management of this disease. A recent study has provided new

insight into the role of zinc in this disease. In the presence of ATP, zinc (at a relatively low concentration of 20 μM) was shown to restore chloride ion secretion in cellular and animal models of cystic fibrosis. Zinc was proposed to bound to and activated a class of purinergic receptor channels stimulating an increase in intracellular Ca^{2+} ions, which enables the by-passing of the defective cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels and triggering of Ca^{2+}-dependent chloride secretion (Zsembery et al., 2004). This is an important study, not only for its implications for therapy of cystic fibrosis, but also because it shows for the first time that, when applied at the apical surface, extracellular zinc can modulate airway epithelium function. While animal models of airway inflammation are necessary to more fully explore the mechanisms by which zinc supplementation affects parameters such as airway epithelium apoptosis, inflammation, and airway hyper-responsiveness (AHR), ultimately, double-blinded clinical trials of zinc supplementation in asthma are required. Ideally, maintenance of body zinc levels should be achieved by ensuring proper zinc nutrition with a healthy balanced diet containing foods such as red meat, dried beans, dairy products, and whole grains, especially from regions of normal soil zinc concentrations. zinc -fortified cereals are now a major source of zinc for young children (Arsenault & Brown, 2003). However, there is a need for supplementation with zinc when short-term measures need to be put in place quickly. Supplementation has been used with variable success in at least one airway disease, cystic fibrosis (Krebs et al., 2000). Issues to be considered in such trials are the optimal routes of administration and the best forms of zinc supplement to use for the airways. Zinc sulphate and zinc gluconate have been the most widely used oral forms. Oral zinc supplements have proven to be effective in a

number of conditions affecting distant organs and tissues (Pories et al., 1967). Side effects of oral zinc are nausea, dyspepsia, and the unpleasant metallic taste. According to WHO guidelines, the safe upper limits for infants and very young children are 13 mg / day, for older children and adolescents 20–30 mg/day, and for adults 50 mg/day; these are based on an oral dose of 1 mg zinc /kg/day (Arsenault & Brown, 2003). Long-term effects of chronic zinc supplementation, however, can impair Cu and Fe absorption. In rats, a high ratio of ingested zinc to Cu results in hypercholesterolemia but this was not seen in a study of human rheumatoid arthritis patients receiving recommended doses of zinc supplements for long periods (Honkanen et al., 1991). Depressed immunity, especially diminished lymphocyte proliferation, neutrophil chemotaxis, and phagocytosis, was seen in humans receiving very high doses 100–300 mg zinc /day (Shankar & Prasad, 1998). In addition to oral administration, tissue zinc concentrations can be increased locally, as by topical zinc oxide creams for the skin (Salgueiro et al., 2002) and zinc sulphate enemas for the bowel (Chen et al., 1999). The first attempt to introduce zinc into the airways for therapeutic purposes was that by Franklin (1931) who used electrolysis to introduce aqueous zinc sulphate into nasal epithelium and alleviate symptoms in subjects with hay fever. Novick et al. (1997) suggested that oral zinc gluconate lozenges directly increase nasal zinc levels via increased salivary zinc concentrations and subsequent transfer of zinc to the nasal cavity. They proposed that this route mediated the capacity of zinc gluconate to suppress rhinovirus-induced inflammation in the respiratory tract as well as the anecdotally reported beneficial effects of zinc gluconate in allergic rhinitis. In 1977, Cho and his colleagues used zinc sulphate-containing aerosols to prevent allergen-induced bronchoconstriction in sensitized guinea pigs. The safe doses and the

likely side effects of zinc aerosols need to be identified, especially in light of known toxic effects to airways of high does of inhaled zinc in some zinc-related occupations and the loss of olfactory sensations in animals given nasal zinc irrigations (McBride et al., 2003).

Zinc toxicity in the airways

In some mining industries and during galvanization of iron, welding, and manufacture of brass, zinc may reach toxic levels in the air and pose a significant health risk to chronically exposed workers (Merchant & Webby, 2001). zinc toxicity is associated with an acute febrile illness, characterized by chills, fever, and myalgias, which result from an acute respiratory tract inflammation accompanied by bronchial hyper-responsiveness. There is no specific treatment and symptoms usually resolve over a period of 36–48 hr (Fuortes & Schenck, 2000). In one study, more than one in four welders had increased polymorphonuclear leukocytes in their bronchoalveolar lavage fluid, suggestive of pulmonary inflammation (Blanc et al., 1991). There are also reports of zinc fume fever leading to asthma. In one subject, who was chronically exposed to zinc during galvanization, inhalation of a solution of ZnSO4 induced a fall in Forced Expiratory Volume in the first second (FEV1) of 23% (Malo et al., 1993).

A considerable amount of scientific data has extensively demonstrated the essential role played by zinc for human health, these data have been derived from the publications of Salgueiro (Salgueiro et al., 2002), Hambidge (Hambidge et al., 2000), and Prasad (Prasad et al., 1996). Research should now focus on possible links between zinc and genotoxicity as well as data concerning the antioxidant and protective properties of zinc.

Lung Cancer

Cancer is a class of diseases or disorders characterized by uncontrolled division of cells and the ability of these cells to spread, either by direct growth into adjacent tissue through invasion, or by implantation into distant sites by metastasis where cancer cells are transported through the bloodstream or lymphatic system. Cancer may affect people at all ages, but risk tends to increase with age. It is one of the principal causes of death in developed countries. In the USA and other developed countries, cancer is presently responsible for about 25% of all deaths (Jemal et al., 2005). Every year, 0.5% of the population is diagnosed with cancer. Table 2 is the statistics for adults in the United States in 2005.

Table 2. Statistics below are adults in the United States

Male	
Most Common	**Causes of Death**
Prostate Cancer (33%)	Lung Cancer (31%)
Lung Cancer (31%)	Prostate Cancer (10%)
Colorectal Cancer (10%)	Colorectal Cancer (10%)
Bladder Cancer (7%)	Pancreatic Cancer (5%)
Cutaneous melanoma (5%)	Leukemia (4%)

Female	
Most Common	**Causes of Death**
Breast Cancer (32%)	Lung Cancer (27%)
Lung Cancer (12%)	Breast Cancer (15%)
Colorectal Cancer (11%)	Colorectal Cancer (10%)
Endometrical Cancer (6%)	Ovarian Cancer (6%)
Non-Hodgkin Lymphoma (4%)	Pancreatic Cancer (6%)

Derived from (Jemal et al., 2005)

Lung cancer is the malignant transformation and expansion of lung tissue, and is the most lethal of all cancers worldwide, responsible for 1.2 million deaths annually. It is caused mainly by cigarette smoking, and predominantly affected men, but with increased smoking among women, it is now the leading cause cancer death in women (American Cancer Society, 2006).

There are two main types of lung cancer categorized by the size and appearance of the malignant cells seen by a histopathologist under a microscope: non-small cell (80%) and small-cell (roughly 20%) lung cancer. This classification although based on simple pathomorphological criteria has very important implications for clinical management and prognosis of the disease.

Exposure to carcinogens, such as those present in tobacco smoke, immediately causes cumulative changes to the tissue lining the bronchi of the lungs (the bronchial mucous membrane) and more tissue gets damaged until a tumour develops. Most of the causes of lung cancer are enviromental factors: carcinogens such as those in cigarette smoke, radiation exposure, enetic susceptibility, and viral infection.

In the United States, smoking is estimated to account for 87% of lung cancer cases (90% in men and 79% in women). Cigarette smoke contains 19 known carcinogens (Koop, 2006) including radioisotopes from the radon decay sequence, nitrosamine, and benzopyrene. Additionally, nicotine appears to depress the immune response to malignant growths in exposed tissue. The length of time a person continues to smoke as well as the amount smoked increases the person's chances of developing lung cancer. More recent work has shown that, across the developed world, almost 90% of lung cancer deaths are caused by smoking (Peto et al., 1994).

Lung cancer is the second most commonly occurring form of cancer in most western countries, and it is the leading cancer-related cause of death for men and women. In the US, 175,000 new cases are expected in 2006 (National Lung Cancer Partnership, 2005), and 90,700 in men and 80,000 in women. Lung cancer was extremely rare prior to the advent of cigarette smoking. In 1878, malignant lung tumors made up only 1% of all cancers seen at autopsy; this had risen to 10-15% by the early 1900s (Witschi et al., 2001). Case reports in the medical literature numbered only 374 worldwide in 1912 (Adler et al, 2005). Not all cases of lung cancer are due to smoking, but the role of passive smoking is increasingly being recognized as a risk factor for lung cancer, leading to policy interventions to decrease undesired exposure of non-smokers to others' tobacco smoke.

Lung cancer and bronchial epithelial cells

Cancers are mostly derived from epithelial cells that eventually differentiate, and most of the lung cancers deveoloped from the bronchial epithelial cells (Green et al, 1977, Auerbach et al., 1961). Normal human bronchial epithelial cells (NHBE) are therefore a good model for investigating human lung epithelial cell carcinogenesis (Lechner and LaVeck, 1985).

2.5. Growth Arrest and DNA Damage inducible gene (Gadd45)

Introduction

The growth arrest and DNA damage-inducible (Gadd) gene Gadd45 is a member of a group of genes that are induced by DNA damaging agents and growth arrest signals. Gadd45 was initially isolated from Chinese Hamster Ovarian cells (CHO) treated with ultraviolet radiation based on its mRNA inducible expression. However, it was subsequently found to be induced by a wide spectrum of DNA-damaging agents such as methylmethane sulfonate (MMS), nitrogen mustard, melphalan, hydrogen peroxide, hypoxia, many cancer chemotherapeutic drugs, ionizing radiation (IR), growth factor withdrawal, and medium depletion (Fornace et al., 1988; Papathannasiou et al., 1991a; Papathannasiou et al., 1991b; Fornace et al., 1992). After DNA damage, Gadd45 induction is rapid, transient and dose-dependent. Gadd45 induction by certain DNA damage-agents has been detected in a variety of mammalian cells. These cells include multiple mouse cell lines, human fibroblast, human lymphoblast and multiple human tumor lines (Fornace et al., 1992; Fornace et al., 1989).

Gadd45 encodes a conserved 165 amino acid protein with nuclear localization and is widely expressed in normal tissues, particularly in quiescent cellular populations. Expression of Gadd45 protein is regulated throughout the cell cycle and the levels of this protein are highest in G1 phase and greatly reduced during S phase (Carrie et al., 1996; Kearsey et al., 1995). Gadd45 protein has been established to be degraded through the

ubiquitination-mediated proteolysis and may involve the PKCdelta-dependent ubiquitin-proteasome pathway (Leung et al., 2001).

Cell cycle G2/M arrest mediated by Gadd45

Wang et al (1999) firstly showed that microinjection of the Gadd45 expression vector into normal human fibroblasts could block cells at the G2/M transition, which indicated that Gadd45 may cause cell cycle G2/M arrest (Wang et al., 1999). The Gadd45-mediated G2/M arrest was found to depend on wild-type p53, since no arrest was observed either in p53-null Li-Fraumeni fibroblasts or in human cells with abnormal p53.

Gadd45 and cancer

Gadd45 is closely associated with genomic instability and tumorigenesis. The importance of Gadd45 in maintenance of genomic stability has clearly been reflected by the phenotypic alterations observed in Gadd45-null mice. Studies have reported that disruption of endogenous Gadd45 has a substantial impact on genomic instability and growth control (Hollander et al., 1999). Gadd45-null mice generated by gene targeting exhibit severe genomic instabilities, which are exemplified by aneuploidy, chromosomal aberrations, gene amplification, centrosome amplification, abnormal mitosis, and cytokinesis (Hollander et al., 1999). Most strikingly, mice lacking the Gadd45 gene are more susceptible to DNA damage-induced tumors, including carcinogenesis induced by UV radiation, ionizing radiation, and dimethylbenzanthracene (DMBA) (Hollander et al.,

1999; Hollander et al., 2001; Hildesheim et al., 2002). After treatment with carcinogens, nearly twice as many Gadd45-null than wild-type mice had multiple tumors, and three times as many had multiple malignant tumors, suggesting that inactivation of Gadd45 may be associated with the development of malignancy. Interestingly, in DMBA-treated Gadd45-null mice, a markedly increase in female ovarian tumors and male hepatocellular tumors has been shown, but an increased in vascular tumors in both sexes with defective Gadd45 has been observed (Hollander et al., 2001). Most recently, Gadd45 has been found to regulate matrix metalloproteinases, whose activity promotes cell migration and invasion (Hildesheim et al., 2004). Thus, in addition to its role in tumorigenesis, Gadd45 may also contribute to tumor progression. Most carcinogens are genotoxic agents that produce different types of DNA damage, enhanced tumorigenesis and tumor progression in Gadd45-null mice may result from their decreased DNA repair, disrupted cell cycle checkpoints, impaired signaling pathways and loss of normal growth control.

Gadd45 regulatory pathway: p53-dependent and p53-independent

p53-dependent

The signaling pathways that regulate Gadd45 expression after genotoxic stress is complex and may involve different mechanisms according to various types of DNA damaging agents and different cellular genetic statuses. Upon DNA damage, p53 protein has been established to be stabilized and post-translationally modified probably though phosphorylation–acetylation cascade (Oren et al., 1999; Prives et al., 1999). The activated p53 in turn, up-regulates many target genes that may play roles in different aspects of cellular response (El-Deiry et al., 1998). Interestingly, Gadd45 is the only member of the

Gadd gene group that is frequently inducible by ionizing radiation in human cells with wild-type (wt) p53. After cellular exposure to IR, Gadd45 mRNA can be transcriptionally activated by p53 protein (Kastan et al., 1992; Zhan et al., 1994). Disruption of endogenous p53 by introduction of HPVE6, a p53 inhibitor that associates with p53 protein and promotes p53 degradation, or dominant mutant p53 into cells, results in a substantial reduced Gadd45 induction following IR (Zhan et al., 1996). Moreover, over-expression of MDM2, a p53-targeted gene that forms an auto-regulatory feedback loop and keeps p53 under negative control, also abolishes IR-induction of Gadd45 (Chen et al., 1994). These findings are further supported by the demonstrations that mouse embryo fibroblasts (MEFs), derived from mice where both p53 alleles had been abolished, do not exhibit Gadd45 induction by IR as compared to their parental MEFs (Kastan et al., 1992). Therefore, IR-induction of Gadd45 is strictly dependent on normal cellular p53 function.

ATM kinase appears to be involved in the regulation of Gadd45 induction by IR. The response of Gadd45 to IR has been established to be significantly reduced in AT lymphoblast lines as compared with normal lymphoblast cells (Papathanasou et al., 1991). Currently, the connection between Gadd45 and ATM is thought to be via the induction of p53 (Kastan et al., 1992). The p53-regulatory elements in Gadd45 are found to locate at the third intron of the gene. This putative p53-binding motif in the human and hamster Gadd45 gene matches the p53 consensus sequence in 19 of 20 bp in 16 of 20 bp, respectively (Kastan et al., 1992). Using both immunoprecipitation and gel mobility shift assays, wild-type but not mutant p53 protein strongly binds to this conserved element. Similarly, the p53-binding site in the MDM2 gene is also located at the intronic region of

the gene (Chen et al., 1994). In contrast to the Gadd45 and MDM2 genes, two other p53-regulated genes; $p21^{Waf1/Cip1}$, a potent inhibitor of cyclin-dependent kinase, and Bax, a member of the Bcl-2 family that promotes apoptosis, harbor p53-binding motifs in their promoter regions (El-Deiry et al., 1993; Miyashita et al., 1995).

Much of the DNA damage response is localized to a GC-rich motif in the proximal promoter that contains a large motif for WT1, a tumor suppressor and transcription factor that is known to associate with p53 physically and functionally (Maheswaran et al., 1993). Several lines of evidence indicate that p53 protein exists in the WT1-associated complex and has a synergistic effect with WT1 in the induction of the Gadd45 promoter. Employment of a dominant-negative p53 vector or abrogation of WT1 function by an antisense vector markedly reduced the responsiveness of the Gadd45 promoter to genotoxic stress. In addition to a strong p53-responsive motif in the intronic region of the Gadd45 gene, p53 may also contribute to the stress responsiveness of the Gadd45 promoter in the absence of direct DNA binding (Zhan et al., 1996). Because of the lack of functional/typical p53 binding sites in the Gadd45 promoter, the participation of p53 in the transcriptional induction of the Gadd45 promoter is likely through protein–protein interaction between p53 and WT1 (Zhan et al., 1998). This finding has greatly broadened the known role of p53 in its downstream-targeted genes.

In contrast to IR induction of Gadd45, which requires normal cellular p53 function, most DNA damaging agents and growth arrest signals (designated as non-IR treatments) have been found to induce Gadd45 in cells regardless of p53 status (Zhan et al., 1996). For example, MMS, UV or medium starvation can activate Gadd45 transcription in

multiple human tumor lines containing mutated or deleted p53 alleles, and dominant-negative mutant p53 vectors. Disruption of endogenous p53 results in reduced induction of Gadd45 by non-IR stress (Zhan et al., 1996). Therefore, p53 is not required for Gadd45 response to these non-IR agents, but may contribute to these stress responses. Since the promoter of Gadd45 mainly regulates Gadd45 induction by non-IR agents, the effect of p53 on Gadd45's response to non-IR treatments is likely mediated through p53 interaction with WT1 protein (Zhan et al., 1998).

In spite of the well-defined role for p53 in Gadd45 induction, the most recent findings have surprisingly shown that Gadd45 may play a role as an upstream effector in p53 stabilization following DNA damage (Jin et al., 2003; Bulavin et al., 2003). This Gadd45 function requires activation of p38 kinase, but not JNK or ERK kinases (Bulavin et al., 2003. Therefore, Gadd45, along with p38, appears to form a positive feedback signal in the activation of the p53 pathway.

Tong et al. (2001) have recently demonstrated that activation of JNK and ERK pathways, but not p38, is involved in regulating the UV induction of the Gadd45 promoter. Over expression of JNK1, Raf-1, or MEKK1, which are upstream activators involved in the ERK and JNK pathways, was observed to strongly activate the Gadd45 promoter in a p53-independent manner. Interestingly, the MAP kinase activation on the Gadd45 promoter appears to be mediated through the OCT-1, and disruption of the OCT-1 and CAAT1 sites resulted in abrogation of Gadd45 activation following expression of JNK1, Raf-1, or MEKK1 (Tong et al., 2001). Collectively, the involvement

of MAP kinases in the activation of the Gadd45 promoter has highlighted the complexity of the signaling pathways in Gadd45 induction following genotoxic stress.

Interaction of CDK1 with Gadd45

The physiological role of Gadd45 in G2/M arrest is associated with its inhibition of CDK1/cyclin B1, a protein complex required for G2/M transition during cell cycle progression. Importantly, Gadd45 inhibition of CDK1 kinase activity differs from Wee1, Mik1 or Myt1, which act on CDK1 inhibitory phosphorylation. Gadd45 physically interacts with CDK1 kinase, but not cyclin B1, and reduces the CDK1/cyclin B1 complex formation. Such reduction of the CDK1 and Cyclin B1 complex formation results in altered subcellular localization of cyclin B1 and suppression of CDK1/cyclin B1 activity (Zhan et al., 1999; Jin et al., 2000; Jin et al., 2002). Jin et al. (2002) reported that inducible expression of Gadd45 protein reduces protein levels of cyclin B1 in the nucleus, but has no effect on phosphorylation status for Cdc25C and Chk1 (Jin et al., 2002). Therefore, the suppressive property of Gadd45 on CDK1 kinase activity is mainly due to its physical disruption of the CDK1/cyclin B1 complex. In agreement with these observations, increased expression of cyclin B1 can attenuate the Gadd45-mediated G2/M arrest and growth suppression. Taken all together, following DNA damage treatment or growth arrest signals, p53 functions as a transcription factor to up-regulate the expression of Gadd45. Induced Gadd45 protein interacts with CDK1 kinase and in turn reduces the CDK1/cyclin B1 complex formation. This “free” cyclin B1 protein is likely exported from the nucleus and pumped into the cytoplasmic compartment, and

subject to ubiquitination-mediated protein degradation. Through this machinery, Gadd45 inhibits CDK1 kinase activity and arrests cells at the G2/M transition.

The CDK1-binding domain of Gadd45 has been located at the central region of this protein (amino acids 65–84). The CDK1-binding domain of Gadd45 is also required for Gadd45 inhibition of CDK1 kinase activity. Sequence analysis of the central Gadd45 region reveals no homology to inhibitory motifs of known cyclin-dependent kinase inhibitors, suggesting that the CDK1-binding or -inhibitory domain of Gadd45 is a novel motif. Deletion of this central region in the Gadd45 protein abolishes Gadd45-mediated cell cycle G2/M arrest and Gadd45-induced growth suppression as well, indicating that cell cycle G2/M growth arrest mediated by Gadd45 is one of the major mechanisms by which Gadd45 suppresses cell growth (Jin et al., 2000)

Gadd45 has no appreciative direct effect on the control of cell cycle G1-S arrest, and neither significantly inhibits activities of any G1 phase-related cyclin(s)/kinase(s), including cyclin E/CDK2 or cyclin D1/CDK2 (Zhan et al., 1999). Over-expression of cyclin D1, an inhibitor of the Rb-related pathway, does not affect Gadd45-induced cell growth suppression although it greatly blocks $p21^{Waf1/Cip1}$-induced growth inhibition (Zhao et al., 2000).

Gadd45 and DNA repair

One of the attractive issues related to Gadd45's biological roles is how important this protein is in DNA repair. The earlier study by Smith et al. (1994) has shown that

Gadd45 interacts with proliferating cell nuclear antigen (PCNA) and may act as a player in nucleotide excision repair (NER), although this issue is still controversial and needs to be further elucidated (Kearsey et al., 1995; Kazantsev et al., 1995). Blocking Gadd45 by constitutive antisense expression resulted in reduced levels of DNA repair and can sensitize cells to killing by UV radiation or by cis-platinum, both of which produce DNA cross links (Smith et al., 1996; Smith et al., 2000; Smith et al., 2002). Interestingly, evidence reported by Carrier et al. (1999) has demonstrated that Gadd45 can interact directly with the core histones and destabilize histone–DNA complexes following UV radiation (Carrier et al., 1999). These findings imply that Gadd45 can recognize an altered chromatin state, bind to UV-damaged chromatin, and may modulate DNA accessibility to cellular proteins. Using mouse embryo fibroblasts (MEFs) lacking Gadd45, Smith et al. (1994) have further shown that Gadd45 affects chromatin remodeling of templates concurrent with DNA repair, indicating that Gadd45 may participate in the coupling between chromatin assembly and DNA repair. Furthermore, MEFs with disrupted Gadd45 display reduced survival ability after UV radiation and cisplatin treatment as compared to wild-type MEFs (Carrier et al., 1999). Most recently, Gadd45 has been reported to modulate the roles of the fork head transcription factor FOXO3a in the cell cycle G2/M checkpoint and DNA repair process (Tran et al., 2002).

CHAPTER III:

Suppression of Gadd45 alleviates the G2/M blockage and phosphorylation of p53 and p38 in zinc supplemented normal human bronchial epithelial cells

3. 1. ABSTRACT

Gadd45 plays a vital role as cellular stress sensor in the modulation of cell signal transduction in response to the stress. In this study, we demonstrated for the first time that an elevated zinc status, created by culturing cells at optimal plasma zinc concentration attainable by oral zinc supplementation, is cytotoxic for normal human bronchial epithelial (NHBE) cells. Cells were cultured for one passage in different concentrations of zinc: <0.4 μM (ZD) as severe zinc deficient; 4 μM as normal zinc level in culture medium; 16 μM (ZA) as normal human plasma zinc level; and 32 μM (ZS) as the high end of plasma zinc attained by oral supplementation. Inhibition of cell growth, up-regulation of Gadd45 mRNA and protein expression, and blockage of G2/M cell cycle progression were observed in ZS cells. The siRNA-mediated knocking down of Gadd45 was found to relieve G2/M blockage in ZS cells, which indicated that the blockage was Gadd45 dependent. Moreover, the enhanced phosphorylations of p38 and p53 (ser15) in ZS cells were normalized after suppression of Gadd45 by siRNA, implicating that the enhanced phosphorylation of these proteins were Gadd45 dependent in ZS NHBE cells.

3.2. INTRODUCTION

Bronchial epithelial cells are the physical barrier that separates airway connective tissue and smooth muscle from the airway luminal contents. In the absence of intact epithelial barrier, airway luminal may contain harmful substances which could initiate inflammatory reactions in the submucosa (Folkerts et al., 1998; Thompson et al., 1998; Holgate et al., 1999). Exposure to high concentrations of zinc in the air may cause significant health risk (Merchant et al., 2001). Zinc toxicity can cause acute respiratory tract inflammation together with bronchial hyper-responsiveness. Studies have shown that workers in mining industries had increased polymorpho-nuclear leukocytes and incidence of pulmonary inflammation (Blanc et al., 1991). In view of the prevalence and clinical significance of zinc deficiency in human populations, as well as extensive use of zinc supplementation in animal production and to a lesser extend in human populations, we have initiated studies designed to examine the influence of zinc status on the expression of stress inducible gene, the growth arrest and DNA damage-induced gene (Gadd) Gadd45, in Normal Human Bronchial Epithelial (NHBE) cells. NHBE cells have been selected for this study because they are more representative cell population during lung tissue transformation and are considered to be progenitor cells for human bronchial cancer.

Gadd45 was originally identified as mRNA transcript that was rapidly induced in response to UV radiation (Fornace et al., 1989). Gadd45 is an ubiquitously expressed 21kD acidic protein in response to genotoxic agent, and is involved in many biological processes related to maintenance of genomic stability and apoptosis. Gadd45-/- knock-out mice were more susceptible to DNA-damage induced tumors when subjected

to carcinogens (Hollander et al., 1999; Hollander et al., 2001; Hildesheim et al., 2002). Overexpression of Gadd45 in tumor cell lines was found to inhibit cell growth (Zhan et al., 1994). Moreover, Gadd45 induction through tet-off inducible system suppressed cell growth (Jin et al., 2002). Initially, over expression of Gadd45 by microinjecting Gadd45 expression vector was found to induce G2/M cell cycle arrest (Wang et al., 1999). The importance of Gadd45 in G2/M regulation was further supported by findings of the inability of Gadd45 knock-out mice to arrest G2/M cell cycle progression after exposure to UV radiation. In cells under hyperosmolalitive stress, inhibition of cell cycle progression with Gadd45 induction, which is partially dependent on p38 kinase activity (Kultz et al., 1998). Various studies demonstrated a functional association between stress-activated mitogen-activated protein kinase pathway and Gadd45 in response to environmental stresses (Takekawa et al., 1998). Gadd45 induction also depends on p38 activity in other cell types exposed to oxidative stress (Oh-Hashi et al., 2001), flavonoids (O'Prey et al., 2003), or peripheral benzodiazepine receptor-specific ligands (Sutter et al., 2003). Conversely, p38 activity depends on MEKK4 activation mediated by Gadd45 protein in response to an array of stimuli (Kultz et al., 1998; Chi et al., 2004; Lu et al., 2001; Bulavin et al., 2003). Tumor suppressor gene p53 plays an important role in the maintenance of genomic fidelity by controlling cell cycle checkpoints and apoptotic process following cell exposure to genotoxic stress. The dependence of Gadd45 induction on normal cellular p53 function is well established (Carrier et al., 1999). In response to DNA damage, Gadd45 was found to contribute to the stability of p53.

The objectives of our study were to determine the association of increased Gadd45 expression with the blockage of G2/M in zinc supplemented NHBE cells, and to decipher

the precise molecular mechanisms of action by suppressing Gadd45 expression to elucidate its functions in growth arrest and cell cycle progression in normal human cell type. This study provides evidence to show that in response to the adverse effect of zinc supplementation, the enhanced expression of Gadd45 is involved in the blockage of G2/M progression and enhanced phosphorylation of tumor suppressor genes, p53 and p38 in normal human bronchial cells.

3.3. MATERIALS AND METHODS

Cell culture -- NHBE cells were purchased from Cambrex Bio Science (Walkersville, MD). Cells were plated at 3,500 cells/cm^2 in tissue culture dishes containing bronchial epithelial cell growth medium (BEGM), supplemented with 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 52 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 0.5 pg/ml human recombinant epidermal growth factor, and 6.5 ng/ml triiodothyronine without antibiotics, and cultured at 37°C in a 5% CO_2 incubator. Endotoxin-free medium was used (<0.005 endotoxin units/ml). The medium was changed at day 1 and subsequently every 48 h. The cells were grown to 80% confluence for 6 days, and subcultured using trypsin-EDTA at a ratio of 1: 8 at passage 3 for experimental zinc treatment.

A zinc-free BEGM baseline media, in which Cambrex omitted the addition of $ZnSO_4$, was used as the zinc-depleted medium. This medium consisted of Bronchial Epithelial Basal Media (BEBM) supplemented with growth components, and contained residue amounts of zinc (<0.4 μM), as detected by flame atomic absorption spectrophotometry. The zinc-free basal medium of <0.4 μM was used as the zinc-depleted medium (ZD). For the other four treatment groups, zinc was added to the media in the form of $ZnSO_4$ so that the only difference between these media was the zinc concentration. For the zinc-normal medium (ZN) contained 4 μM of $ZnSO_4$, the zinc-adequate medium (ZA) contained 16 μM of $ZnSO_4$, and the zinc-supplemented medium (ZS) contained 32 μM $ZnSO_4$. The ZN medium was used as a comparison to standard culture media and was used as the control group for experiments. The ZA treatment was used as a representative of human plasma zinc levels, and the ZS group

was used to represent plasma zinc levels attainable by oral supplementation in humans. After NHBE cells were subcultured into one of the four corresponding groups, the cells were cultured overnight in ZN media before changing to their respective medium. Cells were then cultured in ZD, ZN, ZA and ZS for 6 days. The cell number was determined by using a hemocytometer, and cell viability was assessed by trypan blue dye exclusion. Cell morphology was evaluated by using a phase-contrast microscope (Olympus, Tokyo, Japan).

Cellular zinc and DNA determination -- The cells were harvested after reaching 80% confluence. Cells were harvested by treatment with trypsin-EDTA for 5 min in 37^{o}C incubator. Both cells and media were collected by scraping from tissue culture dishes. And cell suspensions were then centrifuged at 500 x g for 5 min at 4^{o}C, and cell pellets were washed with phosphate-buffered saline (PBS). Cells were resuspended into 1.5ml PBS and sonicated for two 30 sec intervals. An aliquot of the sonicated cell suspension was used to measure cellular zinc content by flame atomic absorption spectrophotometry (Hitachi, San Jose, CA). Zinc standard solutions (Fisher, Pittsburgh, PA) ranging from 0.05 ppm to 1.0 ppm were used to generate a linear standard curve. The zinc content of the cells was determined based on these zinc reference solutions. In addition, the certified zinc solutions were compared to bovine Liver Standard Reference (U.S. Department of Commerce, National Institute of Standards, Gaithersburg, MD). Appropriate blanks were employed for all measurements. From the same sample, a small aliquot of the sonicated cell suspension was used to measure cellular DNA content using diphenylamine (William et al., 1986). Data were expressed as cellular zinc per microgram

of DNA because of the linear relationship between cellular DNA and cell number we previously established (William et al., 1986).

RNase Protection Assay -- Total RNA was isolated from NHBE cells using the RNAqueous Kit (Qiagen, Valencia, CA), according to manufacturer's instruction and the integrity of the RNA was verified by electrophoresis and quantified by spectrophotometry. The mRNA abundance of human genes including, PARP, NF-κB, TNF-β, p53, Gadd45, p38, p21, Brca1, PCNA, MDM2, were measured by non-radioactive RNase Protection Assay (Pharmingen, San Diego, CA). The human GAPDH probe was also included in the multi-probe and was used as house-keeping gene for normalization. Labeled riboprobes were synthesized using the Non-Radioactive In Vitro Transcription kit with T7 RNA polymerase (Pharmingen, San Diego, CA), and Biotin-dUTP (Roche, Alameda, CA).

RNase Protection Assay (RPA) was performed using the Pharmingen RPA kit. Each sample contained 10 μg of total RNA from NHBE cells, and 2 μg of the multi-riboprobe. The RNA and labeled probes were co-precipitated with ammonium acetate and ethanol, and resuspended in hybridization buffer at 56^{o}C for 16 h. The RNase digestion was performed at 30^{o}C for 45 min, followed by inactivation with proteinase K cocktail, and subsequent precipitation. Protected fragments were separated by Tris-Urea Polyacrylamide Gel Electrophoresis (PAGE) using pre-casting gel from BioRad (Hercules, CA). Control samples were processed without RNase digestion, only full-length probes were applied. No protected bands appeared in controls, in which yeast RNA replaced NHBE RNA indicating that digestion was complete. The PAGE gel resolved protected probes were transferred to nylon membrane and subjected to UV

crosslinking. The biotin-labeled protected cDNA transferred to the membrane were detected by chemiluminescent signal and visualized by X-ray film exposure.

Cell Cycle Analysis -- DNA contents of cells were assayed by fluorescence-activated cell sorting (FACS). NHBE cells were cultured in ZD, ZN, ZA, and ZS media for one passage, trypsinized, washed in PBS (Ca^{2+}, Mg^{2+} free), and fixed in 70% cold ethanol. Cells were stored at 4°C. For staining, cells were collected by centrifugation, and pellets were suspended in 1.0 ml propidium iodide staining solution (50 mg per ml propidium iodide, 100 U per ml RNase in PBS), and incubated at room temperature for 1 h. Staining was quantitated with a FACSCalibur cytometer (Becton Dickinson, San Jose, CA). The cell numbers were acquired with CELLQuestPro software program (Becton Dickinson, San Jose, CA). Cell cycle distribution percentages of stained nuclei were calculated by using Modfit LT software (Verity Software House, Topsham, ME). The calibration standard LinearFlow Green and the DNA QC Particle kit, for verification of instrument performance, were purchased from Molecular Probes (Eugene, OR) and Becton Dickinson, respectively.

Western Blot analysis -- Nuclear and cytoplasmic protein concentrations were determined by using the BCA kit (Pierce). Forty μg of protein were resolved on a 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Germany) by using a mini-transfer system (Bio-Rad, Hercules, CA). Membranes were blocked with 5% nonfat dry milk in PBS-T (10 mM phosphate buffer pH7.3, 137 mM NaCl, 2.7 mM KCL, and 0.1% Tween 20) for

1 h at room temperature, prior to incubation with 1 μg/ml of primary antibody from Santa Cruz Biotechnology (Santa Cruz, CA), in PBS-T containing 5% nonfat milk at 4°C overnight. Membrane was then washed three times with PBS-T and blotted with a secondary antibody conjugated with horseradish peroxidase (Santa Cruz, Santa Cruz, CA) at room temperature for 1 h, followed by three washes in PBS-T. The protein was visualized by using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Anti-Gadd45, anti-phospho-p53, anti-phospho-p38, anti-actin and anti-histone H1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

siRNA transfection --RNA interference of Gadd45 was performed by using a 23 bp (including 2-deoxynucleotide overhang) siRNA duplex with the DNA sequence AAAGTCGCTACATGGATCAAT. A control siRNA specific targeting to the luciferase protein DNA sequence CCACTACCTGAGCACCCAG was used as a non-silencing control. siRNA duplexes were synthesized from Qiagen (Valencia, CA). For cell transfection with siRNA, NHBE cells cultured on culture dishes at 60% confluency, and siRNA (600 pmol) were introduced into the cells using RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Briefly, cells were cultured under different zinc status in cell culture dishes, in the following treatments: ZD-Luciferase, ZN-Luciferase, ZS-Luciferase, ZD-Gadd45, ZN-Gadd45, and ZS-Gadd45. Aliquots of 600pmol of siRNA-Gadd45 or siRNA-Luciferase were diluted with Opti-MEM medium (Invitrogen, Carlsbad, CA), and another solution containing lipofectamine transfection reagent also diluted with Opti-MEM medium. These two mixtures were combined together at room temperature with gentle vortex and incubated

for 20 min followed by addition of 10 ml corresponding medium for the treatment groups. The mixture was then overlaid onto the 60% confluent NHBE cells. After 48 h, cells were harvested for cell cycle and western blot analyses.

Statistical Analysis -- Each experiment was repeated at least three times. Data were expressed as mean ± SEM. Statistical comparisons were carried out by one-way analysis of variance (ANOVA). Means were examined by the Least Significant Difference post hoc analysis (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

3.4. RESULTS

Zinc supplementation markedly reduced cell growth in normal human bronchial cells

In ZD, ZA and ZS cells, cell growth as measured by DNA content per plate, was found to be reduced to 83%, 79%, and 67%, respectively, of the ZN cells **(Fig.3.1A)**. The culture of cells in zinc depleted medium resulted in significant 18% reduction of cellular zinc as compared to control ZN cells **(Fig.3.1B)**. Moreover, cellular zinc level in ZA and ZS cells was 150% and 300% of that of ZN control cells, respectively. Thus cell growth was reduced both by the low-zinc and high-zinc status, particularly in ZS cells. Furthermore, a dose-dependent elevation in cellular zinc content was observed as the zinc concentration in the media was increased.

Gadd45 mRNA abundance was up-regulated in ZS NHBE cells

Gadd45 mRNA abundance was up-regulated in ZA and ZS NHBE cells to 149±11% and 185±5%, respectively, of ZN control cells (100±1%) **(Fig.3.2**). Although the mRNA abundance in ZD cells appeared to be lower than ZN cells, the difference was not significant. Thus, in ZA and ZS cells, the marked elevations in *Gadd45* mRNA levels were associated with increases in cellular zinc levels and reduction in cell growth.

Gadd45 protein level was markedly elevated in ZS NHBE cells

In ZS cells, western blot analysis indicated an about 5-fold increase in cytoplamic and a 4-fold increase in nuclear Gadd45 proteins when compared with the ZN cells **(Fig.3.3)**. The cytoplasmic and nuclear Gadd45 protein levels were increased 1.4-fold and

1.6-fold, respectively, in ZD cells; as well as 2.2-fold and 1.3-fold, respectively, in ZA cells, as compared to ZN control cells. These findings indicated that the magnitude of increases in Gadd45 protein among the treatment groups was higher than the increases in mRNA levels, especially for the ZS group. Thus, there may be additional posttranscriptional regulation involved in the enhancement of the protein stability of Gadd45. Because the depression of cell growth induced by an upregulation of Gadd45 protein is well established, the marked elevation in Gadd45 protein in ZS cells may contribute to the observed reduction in cell growth.

Zinc supplementation delayed G2/M cell cycle progression in NHBE cells

To uncover the mechanism responsible for the marked cell growth reduction in ZS cells, we next examined the cell cycle progression by flow cytometry. A marked delay in G2/M cell cycle progression was observed in ZS NHBE cells (24.39±0.53%) when compared to ZN cells (13.15±0.12%) **(Fig.3.4)**. The delay was smaller in magnitude in ZA cells, with 15.59±0.20% of cells in G2/M **(Fig.3.4)**. In contrast, no change was observed in ZD cells, with 12.98±0.60% cells in G2/M, when compared to ZN cells **(Fig.3.4)**.

siRNA mediated gene silencing knocked down Gadd45 protein expression

The approach of siRNA-mediated gene silencing of Gadd45 was used to establish whether the knock down of the marked elevation of Gadd45 in ZS cells would normalize the delay in G2/M cell cycle progression. After Gadd45 siRNA tranfection, the Gadd45 protein levels were knocked down to similar levels in all Gadd45 siRNA transfected zinc

treatments, ZD-Gad (56±14%), ZN-Gad (44±10%) and ZS-Gad (53±8%), as compared to ZN-C controls (100±9%) **(Fig. 3.5)**.

siRNA mediated Gadd45 knocked down abrogated the blockage in G2/M cell cycle progression and triggered apoptosis in ZS NHBE cells

The blockage in G2/M cell cycle progression was partially abrogated in ZS-Gad group. Similarly, ZD-Gad and ZN-Gad showed the same amount of cells in G2/M after siRNA transfection **(Fig.3.6)**. Importantly, the knocked down of Gadd45, resulted in a mild form of apoptosis which was similar among the ZD-Gad, ZN-Gad and ZS-Gad treatment groups **(Fig.3.6)**.

siRNA mediated suppression of Gadd45 depressed the enhanced phosphorylation of p53 at position ser 15 and phosphorylation of p38 in ZS NHBE cells

Phosphorylated p53 (ser 15) **(Fig.3.7)** and phosphorylated p38 **(Fig.3.8)** protein levels in ZS cells were induced to around 2-fold and 2.5-fold, respectively, higher than ZN cells. Interestingly, after the suppression of Gadd45 mediated by siRNA, these two induced phosphorylated proteins were decreased to around the same level of ZN control cells in all siRNA (ZD-Gad, ZN-Gad, ZS-Gad) treatment groups. These findings suggest that regulation of these phosphorylated proteins was Gadd45 dependent.

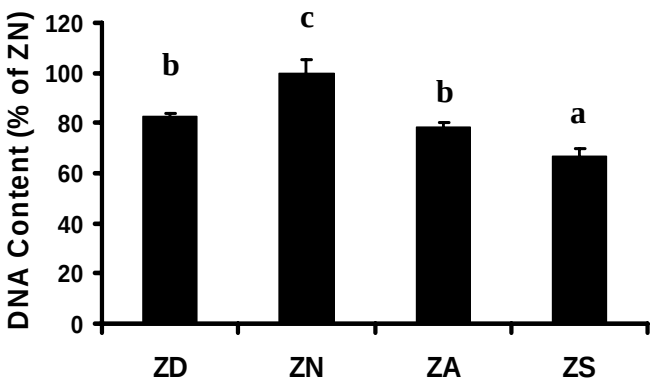


Fig. 3.1A. Zinc supplementation reduced cell growth in NHBE cells.

DNA content per plate in normal human bronchial epithelial (NHBE) cells after cultured in zinc-deficient (ZD, <0.4 μM zinc), zinc-normal (ZN, 4.0 μM zinc), zinc-adequate (ZA, 16.0 μM zinc), and zinc-supplemented (ZS, 32.0 μM zinc) media for one passage. DNA content was determined by diphenylamine method. Values are means ± SEM from three experiments.

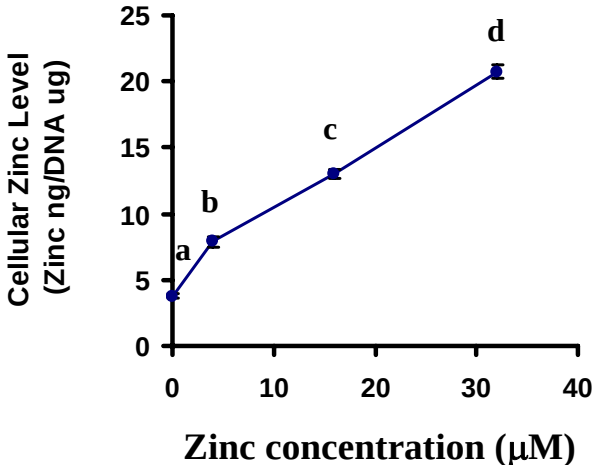


Fig. 3.1B. Cellular zinc level in NHBE cells.

Cellular zinc level in NHBE cells treated with different concentrations of zinc (ZD, ZN, ZA and ZS) for one passage. Cellular zinc was measured by flame atomic absorption spectrophotometry. Values are means ± SEM from three experiments. Means with different letter are significantly different ($p < 0.05$).

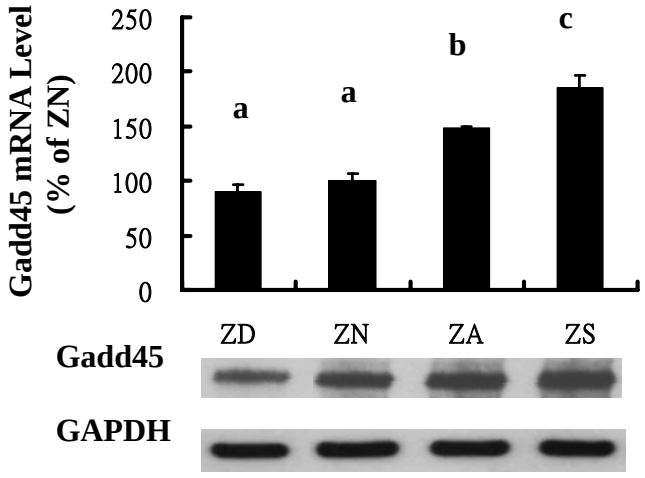


Fig. 3.2. Gadd45 mRNA abundance was up-regulated in ZS NHBE cells.

Cells were cultured in ZD (<0.4 μM zinc), ZN (4.0 μM zinc), ZA (16.0 μM zinc), and ZS (32.0 μM zinc) media. Cells were cultured for 1 passage in BEGM with zinc added as a supplement to the ZD medium. Gadd45 mRNA level was measured by RNase Protection Assay (RPA). RNase protection products were separated on a polyacrylamide gel and quantitated by laser densitometry. GAPDH was used as an internal reference, and values are expressed as a percentage of ZN controls. Representative samples from each treatment group are shown below the bar-graph. Values are means ± SEM from three experiments. Different letters indicate significantly different means, $p < 0.05$. Treatments with the same letters indicate no significant difference.

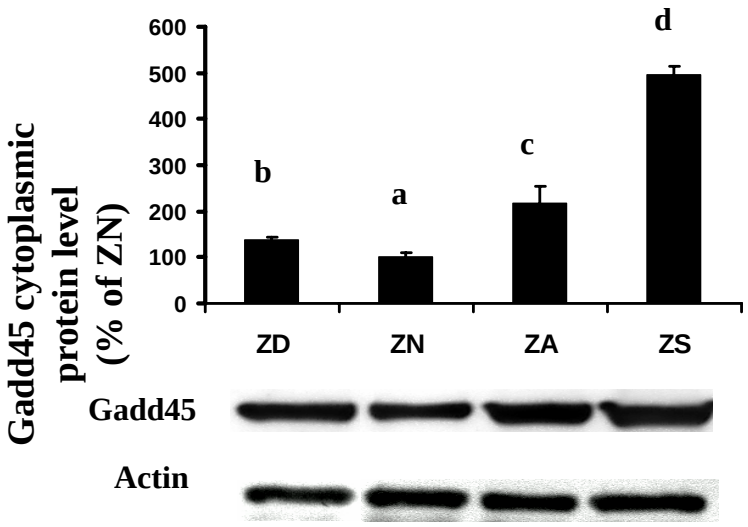


Fig. 3.3A. Relative cytoplasmic Gadd45 protein levels in NHBE cells.

Cells were cultured in ZD (<0.4 μM zinc), ZN (4.0 μM zinc), ZA (16.0 μM zinc), and ZS (32.0 μM zinc) media. Cells were cultured for 1 passage in BEGM with zinc added as a supplement to the ZD medium. Nuclear and cytoplasmic protein extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and incubated with anti-Gadd45 antibody. Cytoplasmic samples were probed with antibodies against Actin for normalization. Autoradiography was visualized using enhanced chemiluminescence and quantitated by densitometry. Values are expressed as a percentage of ZN controls. Representative samples, from each treatment group, are shown below the bar graph. Values are means ± SEM from 3 separated experiments. Different letters indicate significantly different means, $p < 0.05$.

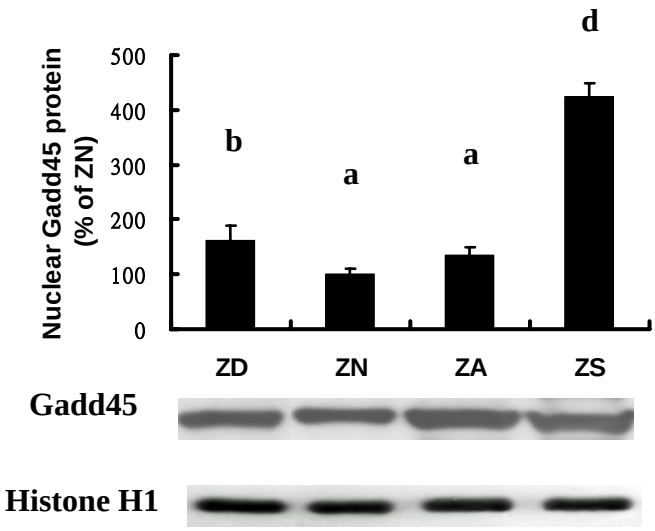


Fig. 3.3B. Relative nuclear Gadd45 protein levels in NHBE cells.

Cells were cultured in ZD (<0.4 μM zinc), ZN (4.0 μM zinc), ZA (16.0 μM zinc), and ZS (32.0 μM zinc) media. Cells were cultured for 1 passage in BEGM with zinc added as a supplement to the ZD medium. Nuclear and cytoplasmic protein extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and incubated with anti-Gadd45 antibody. Nuclear samples were probed with antibodies against Histone H1 for normalization. Autoradiography was visualized using enhanced chemiluminescence and quantitated by densitometry. Values are expressed as a percentage of ZN controls. Representative samples, from each treatment group, are shown below the bar graph. Values are means ± SEM from 3 separated experiments. Different letters indicate significantly different means, $p < 0.05$.

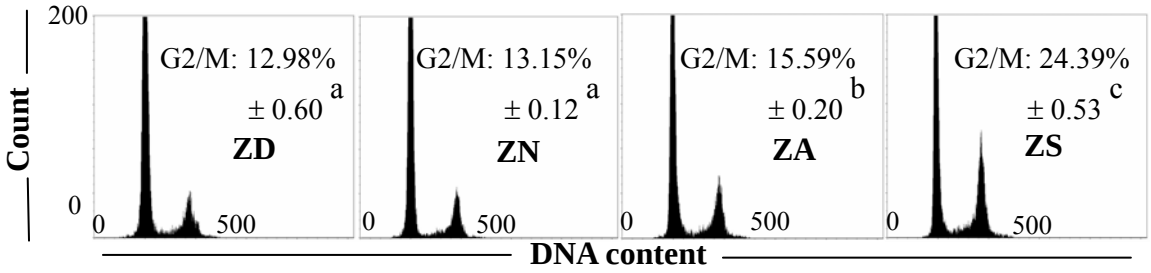


Fig. 3.4. Zinc supplementation blocked G2/M progression in NHBE cells.

Cell cycle analysis of NHBE cells was assayed by flow cytometry using a FACScalibur cytometer. Cells were cultured in ZD, ZN, ZA, and ZS media for one passage. Washed cells were fixed in ethanol and stained with propidium iodide for DNA content. Flow cytometric data files were collected and analyzed using the CELLQuest program. Cell cycle distribution percentages of stained nuclei were calculated using Modfit LT software. The calibration standard LinearFlow Green and the DNA QC particle kit were used for verification of instrument performance.

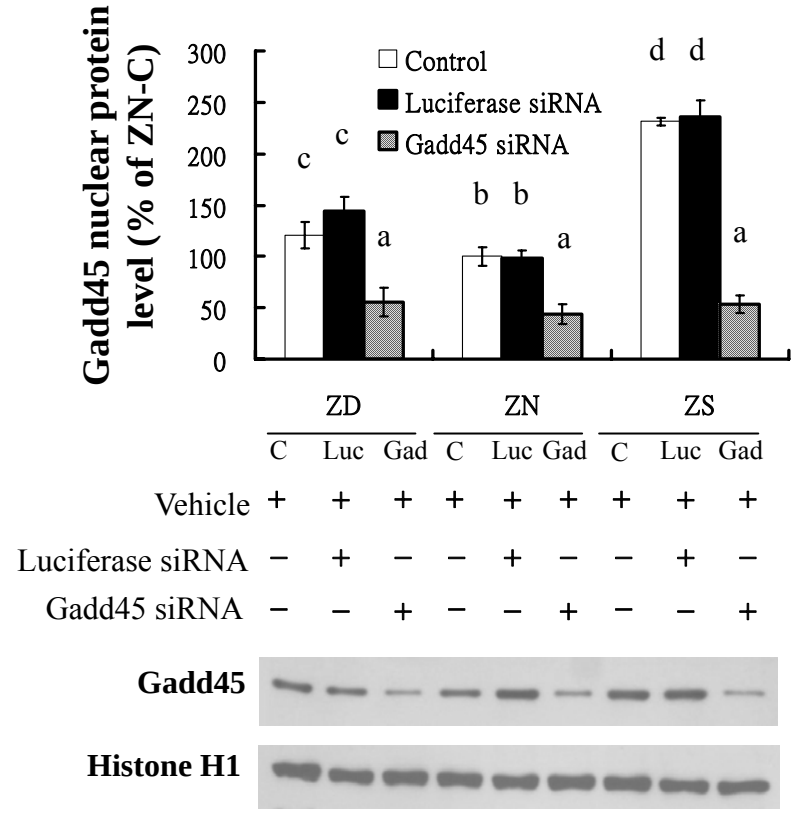


Fig. 3.5. siRNA mediated gene silencing knocked down Gadd45 protein expression

Nuclear Gadd45 protein levels are presented as percentage of ZN-C (control in ZN group). NHBE cells were transiently transfected with either siRNA targeting Gadd45 (Gad) or Luciferase (Luc) in the treatment groups (ZD-Gad, ZN-Gad, ZS-Gad, ZD-Luc, ZN-Luc and ZS-Luc). Control groups (ZD-C, ZN-C and ZS-C) were transfected with lipofectamine only (vehicle). Nuclear Gadd45 protein levels were knocked down by siRNA targeting Gadd45 in ZD-Gad, ZN-Gad and ZS-Gad treatment groups. Representative samples, from each treatment group, are shown below the bar graph. Values are means ± SEM from 3 separated experiments. Different letters indicate significantly different means, $p < 0.05$.

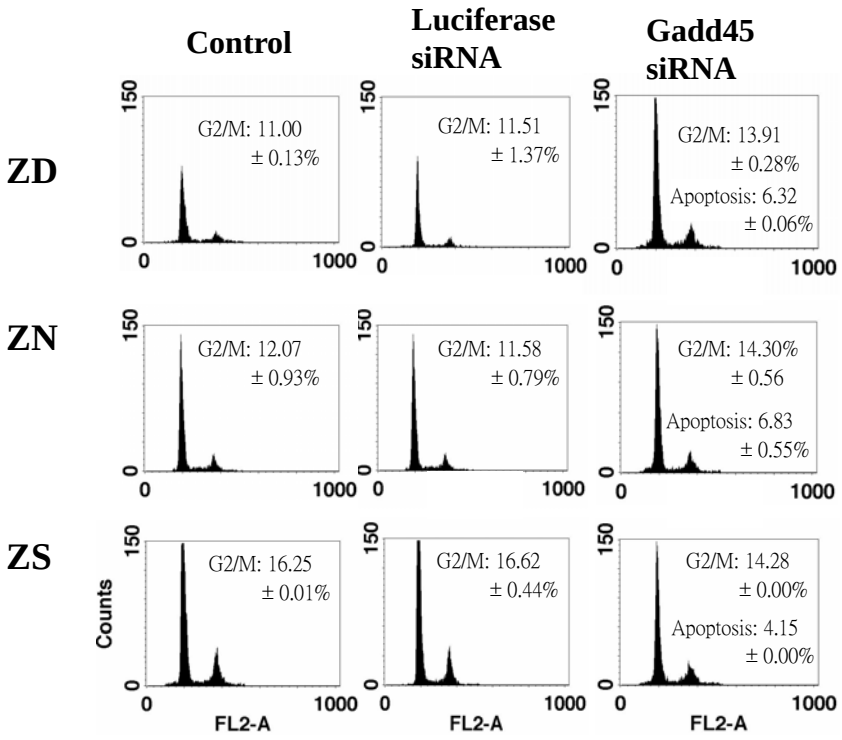


Fig. 3.6. siRNA mediated Gadd45 knocked down released the delay in G2/M cell cycle progression in ZS NHBE cells

Cell cycle analysis was performed after siRNA transfection. NHBE cells were transient transfected with either siRNA targeting Gadd45 (Gad) or Luciferase (Luc) in the treatment groups including ZD-Gad, ZN-Gad, ZS-Gad, ZD-Luc, ZN-Luc and ZS-Luc. Cells were harvested and washed, followed by fixation in ethanol, and stained with propidium iodide for DNA content. Flow cytometric data files were collected and analyzed using the CELLQuest program. Cell cycle distribution percentages of stained nuclei were calculated using Modfit LT software.

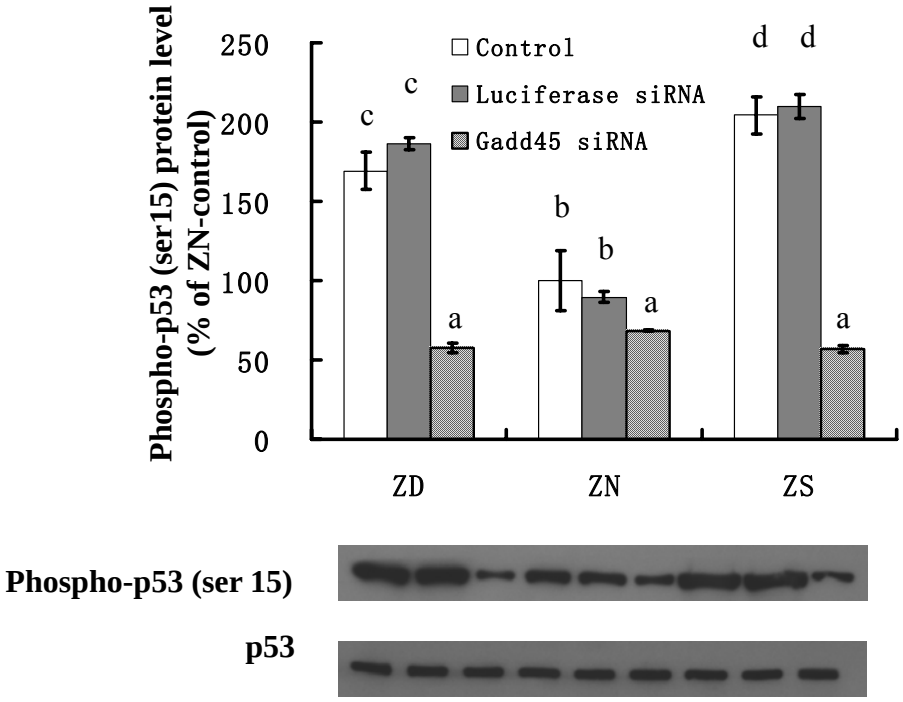


Fig. 3.7. Phospho-p53 (ser15) protein levels were depressed after knocked down of Gadd45 by siRNA

NHBE cells were transiently transfected with either siRNA targeting Gadd45 (Gad) or Luciferase (Luc) in the treatment groups (ZD-Gad, ZN-Gad, ZS-Gad, ZD-Luc, ZN-Luc and ZS-Luc). Control groups (ZD-C, ZN-C and ZS-C) were transfected with lipofectamine only (vehicle). After 48 hours, cells were lysed and analyzed. Total cell extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and probed with anti-phospho-p53 (ser15) antibody. Autoradiography was visualized using enhanced chemiluminescence and quantitated by densitometry. Values are expressed as a percentage of ZN controls. Representative samples, from each treatment group, are shown below the bar graph. Different letters indicate significantly different means, $p < 0.05$.

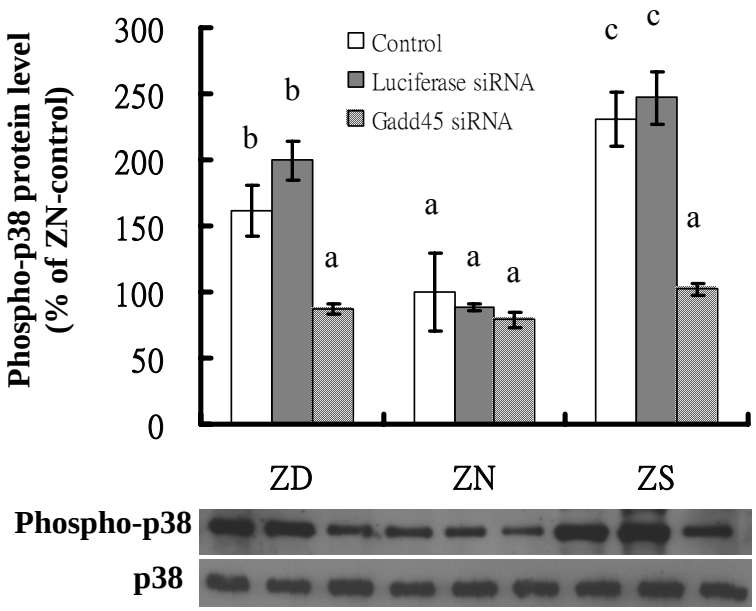


Fig. 3.8. Phospho-p38 protein level was affected after knocked down of Gadd45 by siRNA

NHBE cells were transiently transfected with either siRNA targeting Gadd45 (Gad) or Luciferase (Luc) in the treatment groups (ZD-Gad, ZN-Gad, ZS-Gad, ZD-Luc, ZN-Luc and ZS-Luc). Control groups (ZD-C, ZN-C and ZS-C) were transfected with lipofectamine only (vehicle). After 48 hours, cells were lysed and analyzed. Total cell extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and probed with anti-phospho-p38 antibody. Autoradiography was visualized using enhanced chemiluminescence and quantitated by densitometry. Values are expressed as a percentage of ZN controls. Representative samples, from each treatment group, are shown below the bar graph. Values are means ± SEM from 3 separated experiments. Different letters indicate significantly different means, $p < 0.05$.

3.5. DISCUSSION

In this report, we provide evidence that G2/M blockage found in physiological range of zinc supplementation in NHBE cells is associated with upregulation of Gadd45 expression. The administration of small interference RNA targeting Gadd45 abrogated the G2/M blockage in zinc supplemented NHBE cells. The silencing of Gadd45 also suppressed the phosphorylated-p53 (ser 15) and phosphorylated-p38 levels, which indicate that Gadd45 may contribute to the enhanced phosphorylation of p53 and p38 in response to the stress induced by zinc supplementation in NHBE cells.

Zinc supplementation inhibits normal human bronchial cells proliferation

Our studies were designed to investigate the influence of zinc depletion and supplementation on the cell signaling and cell cycle regulation by Gadd45 in normal human bronchial epithelial cells (NHBE). In zinc supplemented NHBE cells, cellular zinc was threefold higher than that of ZN control group **(Fig.3.1B)**, and cell growth as measured by cell number was decreased to 60% of ZN **(Fig.3.1A)**. Our study is the first to demonstrate that zinc supplementation, within the physiological range, is cytotoxic and reduces the growth of NHBE in primary culture. Thus, high physiologic level of zinc also imposes adverse effect in normal cells. Controversy towards the efficacy of zinc supplementation in the prevention of cancer growth has been reported. Several studies have indicated that high cellular zinc levels inhibit cancer cell growth (Bataineh et al., 2002; Feng et al., 2000; Uzzo et al., 2002). Moreover, studies also reported that treatment with high level of zinc not only inhibited proliferation cancer cells but also induced G2/M arrest (Jaiswal et al., 2004; Liang et al., 1999). However, epidemiological study has

reported an increase in risk for cancer development with usage of high level of zinc supplement (Leitzmann et al., 2003). Even though zinc supplementation can inhibit cancer cell proliferation, the cytotoxic effect of zinc (90 and 900 μmol/L) resulting in the induction of DNA fragmentation has been observed in human primary liver cells by induction of DNA fragmentation (Paramannantham et al., 1996).

Upregulation of Gadd45 is associated with cell growth suppression in ZS NHBE cells

Our results demonstrate that cell growth suppression was accompanied by marked induction of Gadd45 expression, suggesting that Gadd45 may be the main regulator in cell growth inhibition in zinc supplemented NHBE cells. This hypothesis is supported by numerous findings regarding the function of Gadd45 reported by other research group. Several findings have established that over-expression of Gadd45, by transient transfection and tet-off inducible system, substantially inhibits cell growth in multiple tumor lines (Zhan et al., 1994; Jin et al., 2002). In addition, Gadd45 has been established to be downstream of p53-dependent pathway in cells harboring functional p53 or null-p53. To determine whether Gadd45 is the single regulator of p53-dependent pathway in response to cellular stress, experiment is in progress, and $p21^{WAF/CIP1}$ is the candidate gene to be studied. Although Gadd45 reveals a similar growth suppressive property as $p21^{WAF/CIP1}$, it does not utilize Rb-related pathways and may act differently to exert its growth-suppressive function.

G2/M blockage was found in ZS NHBE cells

In our studies, cell cycle analysis by flow cytometry showed there was G2/M blockage in ZS cells **(Fig. 3.4).** In ZS cells, G2/M blockage was accompanied by three to fivefold induction of nuclear and cytoplasmic Gadd45 proteins levels, respectively **(Fig 3.3A & 3.3B)**, and by two-fold increase in mRNA expression **(Fig.3.2)** as compared to ZN control cells. These results suggest that zinc supplementation may cause cellular stress which induces Gadd45 expression. With the increase in Gadd45 expression in ZS cells, G2/M blockage occurs to delay the transition and hinder cell growth. The underlying mechanism by which Gadd45 blocks the G2/M cell cycle progression needs to be further investigated. This probably is due to the binding of Gadd45 to CDK1, which decreases CDK1 kinase activity, and hinders the cell progression from G2 to M. High dosage of zinc has been reported to induce DNA fragmentation in human cells, implying that DNA damage may occur in high zinc supplementation. The molecular mechanism by which stress signals arrest at G2/M checkpoint is still not fully established. The cell cycle control system is based on the CDK and the cyclins. Cyclin B1 undergoes a cycle of synthesis and degradation with each division cycle, is capable of binding to CDK1. This dimer forms the M phase-promoting factor (MPF) during G2 and is required for entry into mitosis. Gadd45 has been shown to bind Cdk1 and inhibit its activity or alter the subcellular level of Cyclin B1 in human cells when exposed to stresses like ionizing radiation or ultra violet radiation (Jin et al., 2002; Zhan et al., 1999; Jin et al., 2000). Gadd45 induces G2/M arrest through p53-dependent and independent pathways by decreasing Cdk1 kinase activity and changing subcellular Cyclin B1 levels in many cell types when exposed to stresses (Wang et al., 1999; Mullan et al., 2001; Maeda et al.,

2002; Jiang et al., 2003; Mak et al., 2004). The effect of Gadd45 on the G2/M transition may be due to its ability to reduces complex formation of Cyclin B1and CDK1 and inhibit the activity of CDK1/Cyclin B1 in vitro (Zhan et al., 1999; Jin et al., 2000). In the present study, no G1 arrest was observed in our study. This is consistent with data from another study which showed that Gadd45 did not efficiently inhibit CDK2/Cyclin E nor caused G1 arrest when microinjected (Zhan et al., 1999). However, observed G2/M blockage may require p53 if Gadd45 acts directly on CDK1/Cyclin B1. One explanation is that Gadd45 may cooperate with other downstream targets of p53, such as p21, whose constitutive expression is lost upon deletion of p53 (Tang et al., 1998).

The upregulation of Gadd45 accounts for the G2/M blockage in ZS NHBE cells

To show the involvement of Gadd45 in G2/M cell cycle regulation in zinc supplemented NHBE cells, we initiated the small interfering RNA targeting Gadd45 experiment to knock down Gadd45. The success of knocking down Gadd45 was confirmed by the marked reduction in Gadd45 protein level **(Fig.3.5)**. Gadd45 protein was knocked down to 40-50% of ZN control group (ZN-C). Consistent with reports from other research groups, which studied the effect of knocking out Gadd45, cell cycle analysis of NHBE cells by flow cytometry indicated a decrease in G2/M blockage in ZS cells after targeting Gadd45 siRNA (ZS-Gad) than in ZS control groups (ZS-C & ZS-Luc; **Fig.3.6** & Table 2). Thus, the up-regulated Gadd45 expression may partially account for the G2/M blockage in zinc supplemented cells. After the knock down of Gadd45, a small but similar extend of apoptosis was observed among all zinc treatment groups in NHBE cells. This is because the suppression of Gadd45 can no longer protect cells from

apoptosis under the toxic status induced by zinc supplementation. The reason for the abrogated G2/M blockage not normalizing to level of ZN (ZN-C) cells may be due to another signaling pathway independent of Gadd45 that regulates G2/M transition, which may include p38. This project provides evidence that Gadd45 plays a direct role in the G2/M cell cycle checkpoint in zinc supplemented NHBE cells. In addition the findings are consistent with other studies which reported the correlations of Gadd45 upregulation with G2/M arrest in human fibroblasts (Wang et al., 1999; Maeda et al., 2002; Smith et al., 1996).

Possible pathways regulate Gadd45 in G2/M blockage in ZS NHBE cells

Gadd45 was shown to be a direct target gene of p53 tumor suppressor gene, and plays a vital role in cell cycle progression. Gadd45 can be regulated either by p53-dependent or –independent pathway in response to stress. In this study, the p53 mRNA and protein expressions exhibited similar increases in ZS NHBE cells (data not shown). Moreover, the silencing of Gadd45 suppressed the phosphorylation of p53 at position serine 15 which is required for activation of p53, suggesting that Gadd45 can behave as an upstream activator of p53 phosphorylation process through another mechanism. Our findings on the relationship of Gadd45 and p53 indicated that the upregulation of Gadd45 enhanced the phosphorylation of p53. Similarly, other studies have shown that upregulation of Gadd45 is capable of enhancing p53 protein level through a positive feedback loop by binding to MTK1 and activating p38 indirectly (Jin et al., 2003; Takekawa et al, 1998). Moreover, studies have established that Gadd45 can bind with PCNA (Ph-tani-Fujita et al., 1998; Kearsey et al., 1995; Smith et al., 1994) and

$p21^{WAF/CIP1}$ (Vairapandi et al., 1996) which are downstream regulators of p53. Furthermore, chromatin immunoprecipitation studies have shown that p53 preferentially binds to the responsive element in the promoters of p21 and GADD45 but not to promoters of other target genes that recruited p53 following DNA damage (Jackson et al., 2006). Therefore, the observed enhanced Gadd45 expression possibly may act in a p53-dependent pathway in the G2/M blockage in the ZS NHBE cells.

Another possible regulatory pathway responsible for the upregulation of Gadd45 in ZS NHBE cells is the interaction of Gadd45 protein with the activated p38 MAPK directly or indirectly. Indirectly, Gadd45 can bind to N-terminal of MTK1 and disrupt MTK1 itself N-C interaction. This Gadd45-binding induces MTK1 N-C dissociation, dimerization, and autophosphorylation and leads to activation of MAPK, including p38 (Miyake et al., 2007). Directly, there is on p38 one interacting domain in the region of amino acids 71 to 96 in Gadd45. And this region is required to activate p38 in the presence of H-ras oncogene (Bulavin et al., 2003). In our study, we demonstrated that silencing Gadd45 suppressed p38 phosphorylation, suggesting that the activation of p38 is mediated through Gadd45 protein. The Gadd45 protein may directly bind to p38 upstream regulator, such as MTK1 and trigger the phosphorylation of p38 in ZS NHBE cells. The activated p38 then binds to downstream regulator, p53, and activates its function, and consequently arrests the NHBE cells in G2/M. Apart from interacting with Gadd45 in stress induced G2/M arrest, p38 also acts independently of p53 pathway by shuttling phosphatase CDC25B from nucleus to cytoplasm and recruited 14-3-3 to bind to CDC25B (Bulavin et al., 2001). CDC25B is required to activate CDK1 by removing two phosphate groups and it is required for entry into mitosis.

In summary, the present results presented above provide novel findings that zinc supplementation, in a physiologically relevant system, exerted adverse effect in normal cells, markedly elevated Gadd45 expression, inhibited G2/M progression, and enhanced phosphorylation of p53 (ser 15) and p38 in NHBE cells, and the related mechanism responsible for the cell cycle arrest in normal human cells. A better understanding of the mechanisms involved in the protection of normal cells from zinc cytotoxicity is essential for the identification of potential targets, such as Gadd45, p53 and p38, and may contribute to the development of future therapeutic approaches for the treatment of human lung cancer.

CHAPTER IV:

Up-regulates Gadd45 expressions and promoter activity, impairs CDK1/Cyclin B1 complex formation and delays G2/M are p53 dependent in zinc supplemented human bronchial epithelial cells

4.1. ABSTRACT

Zinc supplementation has been established to be beneficial in the human body including enhanced immune system and normalization of stunted growth. However, zinc supplementation in physiological range was found to be cytotoxic in cell culture system in this study. p53 plays a central role in the modulation of cell signal transduction in response to the stress from DNA damage, hyporexia and oncogene activation. In this study, we demonstrated for the first time that an elevated zinc status, created by culturing cells at optimal plasma zinc concentration attainable by oral zinc supplementation, is cytotoxic for normal human bronchial epithelial (NHBE) cells. Cells were cultured for one passage in different concentrations of zinc: <0.4 μM (ZD) as zinc deficient; 4 μM as normal zinc level in culture medium; 16 μM (ZA) as normal human plasma zinc level; and 32 μM (ZS) as the high end of plasma zinc attainable by oral supplementation. Inhibition of cell growth, up-regulation of p53 mRNA and protein expressions, and blockage of G2/M cell cycle progression were observed in ZS cells. The dependency on p53 in the modulation of Gadd45 expression was established by applying p53 protein inhibitor, Pifithrin, to inhibit p53 transactivation activity. The elevated Gadd45 protein level and Gadd45 promoter activity caused by zinc supplementation were normalized by Pifithrin treatment to the level of ZN control cells, suggesting that the ZS induced p53 is responsible for the enhanced transactivation of Gadd45 promoter.

Furthermore, the reduction in the level of CDK1/Cyclin B1 complex and the G2/M blockage in ZS cells was normalized almost to that of ZN cells by Pifithrin treatment, which indicated that these changes were p53 dependent.

4.2. INTRODUCTION

Bronchial epithelial cells are the physical barrier that separate airway connective tissue and smooth muscle from the airway luminal contents. Exposure to high concentration of zinc in the air may cause significant health risk (Merchant et al., 2001). Zinc toxicity can cause acute respiratory tract inflammation together with bronchial hyper-responsiveness. Studies have shown that workers in mining industries have increased polymorpho-nuclear leukocytes and incidence of pulmonary inflammation (Blanc et al., 1991). In view of the prevalence and clinical significance of zinc deficiency in human populations, as well as extensive use of zinc supplementation in animal production and to a lesser extend in human populations, we have initiated studies designed to examine the influence of zinc status on the expression of tumor suppressor genes, p53, and the growth arrest and DNA damage-induced gene (Gadd) Gadd45, in Normal Human Bronchial Epithelial (NHBE) cells. NHBE cells have been selected for this study because they are more representative of the cell population during lung tissue transformation and are considered to be progenitor cells for human bronchial cancer.

Tumor suppressor gene p53 is the most frequently mutated gene in cancer (Vogelstein and Kinzler, 1992) and has been implicated in maintaining genomic stability by controlling cell cycle checkpoints and apoptosis following genotoxic stress (Kastan et al., 1992; Zhan et al., 1994b; Miyashita and Reed, 1995; Waldman et al., 1995; White, 1996; Levine, 1997). Stresses including UV radiation, alkylating agents and ionizing radiation damage DNA and activated protein, such as p53, which is stabilized and activated as a transcription factor (Kastan et al., 1991; Zhan et al., 1993). Activated p53 transactivates its downstream genes, including Gadd45, p21 and Bax (El-Deiry et al.

1993, Kastan et al., 1992; Zhan et al., 1994a, 1993). Subsequently, cell cycle arrest or apoptosis will prevent the cells from passing damaged DNA to mitotic cells.

Gadd45 was originally identified as a mRNA transcript that was rapidly induced in response to UV radiation (Fornace et al., 1989). Gadd45 is ubiquitously expressed as a 21kD acidic protein in response to genotoxic agents, and is involved in many biological processes related to the maintenance of genomic stability and apoptosis. Gadd45 has been implicated to interact with PCNA, p21, CDK1 and MTK1 (Smith et al., 1994; Kearsey et al., 1995; Zhao et al., 2000; Takekawa and Saito, 1998). Gadd45 also has been established to regulate G2/M arrest in response to genotoxic stress and maintainance of genomic stability. Regulation of Gadd45 induction after DNA damage is complex and may involve both p53-dependent and -independent signaling pathways. Gadd45 induction by IR has been reported to be strictly dependent on normal cellular p53 function. However, Gadd45 induction by UV and MMS does not require p53 although p53 may contribute to the non-IR response of Gadd45 (Kastan et al., 1992; Zhan et al., 1996).

The objectives of our study were to determine whether the increased Gadd45 expression with the blockage of G2/M in zinc supplemented NHBE cells is p53-dependent or p53-independent, to decipher the precise molecular mechanisms responsible for the regulation of Gadd45 expression by p53, and to elucidate Gadd45 functions in growth arrest and cell cycle progression in NHBE cells. This study provided evidence for the first time to show that the upregulation of Gadd45 is dependent on p53 in the delay in G2/M progression, by the application of p53 inhibitor, Pifithrin, which inhibited the transactivation of p53 function, in NHBE cells culture at optimal zinc concentration previously established to be attainable in the plasma by oral

supplementation.

4.3. MATERIALS AND METHODS

Cell Culture and treatment --NHBE cells were purchased from Cambrex Bio Science (Walkersville, MD). Cells were plated at 3,500 cells/cm^2 in tissue culture dishes containing bronchial epithelial cell growth medium (BEGM), supplemented with 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 52 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 0.5 pg/ml human recombinant epidermal growth factor, and 6.5 ng/ml triiodothyronine (as growth supplements) without antibiotics, and cultured at 37°C in a 5% CO_2 incubator. The cells were grown to 80% confluence for 6 days, and subcultured using trypsin-EDTA at a ratio of 1: 8 at passage 3 for experimental zinc treatment.

A zinc-free BEGM baseline medium, in which Cambrex omitted the addition of $ZnSO_4$, was used as the zinc-depleted media. This media consisted of Bronchial Epithelial Basal Media (BEBM) supplemented with growth components, and contained residual amounts of zinc (<0.4 μM), as detected by flame atomic absorption spectrophotometry. The zinc-free basal medium of <0.4 μM was used as the zinc-depleted medium (ZD). For the other four treatment groups, zinc was added to the media in the form of $ZnSO_4$ so that the only difference between these media was the zinc concentration. For the zinc-normal medium (ZN) contained 4 μM of $ZnSO_4$, the zinc-adequate medium (ZA) contained 16 μM of $ZnSO_4$, and the zinc-supplemented medium (ZS) contained 32 μM $ZnSO_4$. The ZN medium was used as a comparison to standard culture media and was used as the control group for experiments. The ZA

treatment was used as a representative of human plasma zinc levels, and the ZS group was used to represent plasma zinc levels attainable by oral supplementation in humans. After NHBE cells were subcultured and assigned into one of the four corresponding groups, the cells were cultured overnight in ZN media before changing to their respective medium. Cells were then cultured in ZD, ZN, ZA and ZS for 6 days. Pifithrin (PFT) was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louise, MO), Chemicals were used as stock solutions in dimethylsulfoxide. PFT was administrated into the cells in the working concentration of 2 μM for 48 h.

RNase Protection Assay -- Total RNA was isolated from NHBE cells using the RNAqueous Kit (Qiagen, Valencia, CA), according to manufacturer's instruction and the integrity of the RNA was verified by electrophoresis and quantified by spectrophotometry. The mRNA abundance of human genes including, PARP, NF-κB, TNF-β, p53, Gadd45, p38, p21, Brca1, PCNA, MDM2, were measured by non-radioactive RNase Protection Assay (Pharmingen, San Diego, CA). The human GAPDH probe was also included in the multi-probe and was used as house-keeping gene for normalization. Labeled riboprobes were synthesized using the Non-Radioactive In Vitro Transcription kit with T7 RNA polymerase (Pharmingen, San Diego, CA), and Biotin-dUTP (Roche, Alameda, CA).

RNase Protection Assay (RPA) was performed using the Pharmingen RPA kit. Each sample contained 10 μg of total RNA from NHBE cells, and 2 μg of the multi-riboprobe. The RNA and labeled probes were co-precipitated with ammonium acetate and ethanol, and resuspended in hybridization buffer at 56^{o}C for 16 h. The RNase digestion was performed at 30^{o}C for 45 min, followed by inactivation with proteinase K cocktail, and

subsequent precipitation. Protected fragments were separated by Tris-Urea Polyacrylamide Gel Electrophoresis (PAGE) using pre-casting gel from BioRad (Hercules, CA). Control samples were processed without RNase digestion, only full-length probes were applied. No protected bands appeared in controls, in which yeast RNA replaced NHBE RNA indicating that digestion was complete. The PAGE gel resolved protected probes were transferred to nylon membrane and subjected to UV crosslinking. The biotin-labeled protected cDNA transferred to the membrane were detected by chemiluminescent signal and visualized by X-ray film exposure.

Gadd45 Promoter Activity Assay

Preparation of Gadd45-Luciferase The Gadd45 promoter construct was a kind gift provided by Dr. Towia Libermann (Zerbini et al., 2004). The promoter region was isolated and inserted into the plasmid pGL3-basic (Promega, Madison, WI) to generate construct pGL3-Gadd45-Luci plasmid. The plasmid was transfected into *E. coli* DH5α competent cells (Invitrogen, Carlsbad, CA) by standard protocol for transformation. The plasmid was prepared by using Wizard PureFection Plasmid DNA purification system from Promega (Madison,WN).

Transient Transfection and Luciferase Assay – NHBE cells were transfected by using Tfx-20 reagent according to the protocol provided by the manufacturer (Promega). NHBE cells, in complete medium, were seeded at a density of 2×10^5 cells/well in 24-well plates and cultured for 4 days in complete medium containing 0, 4.0, or 32.0 μM zinc. Just before transfection, the medium was removed. Transfections were performed in

triplicate with 500 ng of the plasmid DNA containing the wild-type Gadd45 promoter luciferase reporter construct and 10 ng of an internal control plasmid, pRL-SV40 (Promega). One hour after transfection, the transfection medium was changed to their respective media and cultured for 2 more days. Luciferase activity was measured in the Luminometer TD-20/20 (Turner Designs, Sunnyvale, CA) by using the Dual-Luciferase reporter Kit according to recommendations by the manufacturer (Promega). Changes in firefly luciferase activity was calculated and plotted after normalization with changes in renilla luciferase activity in the same sample.

Cell Cycle Analysis -- DNA contents of cells were assayed by fluorescence-activated cell sorting (FACS). NHBE cells were cultured in ZD, ZN, ZA, and ZS media for one passage, trypsinized, washed in PBS (Ca^{2+}, Mg^{2+} free), and fixed in 70% cold ethanol. Cells were stored at 4°C. For staining, cells were collected by centrifugation, and pellets were suspended in 1.0 ml propidium iodide staining solution (50 mg per ml propidium iodide, 100 U per ml RNase in PBS), and incubated at room temperature for 1 h. Staining was quantitated with a FACSCalibur cytometer (Becton Dickinson, San Jose, CA). The cell numbers were acquired with CELLQuestPro software program (Becton Dickinson, San Jose, CA). Cell cycle distribution percentages of stained nuclei were calculated by using Modfit LT software (Verity Software House, Topsham, ME). The calibration standard LinearFlow Green and the DNA QC Particle kit, for verification of instrument performance, were purchased from Molecular Probes (Eugene, OR) and Becton Dickinson, respectively.

Nuclear and Cytoplasmic Extract Preparation -- The NE-PER Nuclear and Cytoplasmic Extraction Reagents and the Halt Protease Inhibitor Cocktail Kits (Pierce Biotechnology, Rockford, IL) were used for nuclear and cytoplasmic extracts preparation according to the manufacturer's instructions, which are based on the method of Smirnova et al. (2000). Nuclear and cytoplasmic extracts were then stored in aliquots at -80°C. Protein concentrations were determined by using the BCA Protein Assay Reagent kit (Pierce, Rockford, IL). Contaminations of nuclear extracts by cytoplasmic proteins or contaminations of cytoplasmic extracts by nuclear proteins, detected by Western blot analysis of Hsp90 or Oct-1, respectively, were routinely found to be less than 5% in our lab.

Western Blot Analysis -- Nuclear and cytoplasmic protein concentrations were determined by using the BCA kit (Pierce). Forty μg of protein were resolved on a 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Germany) by using a mini-transfer system (Bio-Rad, Hercules, CA). Membranes were blocked with 5% nonfat dry milk in PBS-T (10mM phosphate buffer pH7.3, 137mM NaCl, 2.7mM KCL, and 0.1% Tween 20) for 1 h at room temperature, prior to incubation with 1 μg/ml of primary antibody from Santa Cruz Biotechnology (Santa Cruz, CA), in PBS-T containing 5% nonfat milk at 4°C overnight. Membrane was then washed three times with PBS-T and blotted with a secondary antibody conjugated with horseradish peroxidase (Santa Cruz, Santa Cruz, CA) at room temperature for 1 h, followed by three washes in PBS-T. The protein was visualized by using the SuperSignal West Pico Chemiluminescent Substrate (Pierce,

Rockford, IL). Anti-Gadd45, anti-p53, anti-Cyclin B1, anti-actin and anti-histone H1 were purchased from Santa Cruz Biotechnology (Santa Cruz, Santa Cruz, CA).

Immunoprecipitation of CDK1/CyclinB1 – Seventy percentage confluency of cells were harvested, and washed with 10 ml of PBS. Cells were then washed and resuspended in 1 ml of cold lysis Buffer containing 1X Protease Inhibitor Cocktail (Pierce, Rockford, IL). Cells were then placed on ice for 30 min and centrifuged at 10,000 x g for 15 min at 4^{o}C. The supernatant was collected in new microcentrifuge tube. Protein concentration in the whole cell lysates were determined by BicinChoninic Acid (BCA) reagent kit (Pierce). Five hundred μg of proteins from whole cell lysate were added to 50 μl of Protein G (Santa Cruz Biotechnology), washed with cold lysis buffer, and incubated on ice for 1 h. The complex was then centrifuged at 10,000 x g for 10 min at 4°C. Ten μg of Cyclin B1 antibody or rabbit polyclonal antibody (Santa Cruz Biotechnology) were added to the precleared lysate and incubated for 1 h. Protein A/G PLUS agarose (Santa Cruz Biotechnology) was added and incubated at 4°C for overnight on a rocking platform. The immunocomplexes were centrifuged at 10,000 x g for 30 seconds at 4°C and washed five times with 500 μl of lysis buffer. The 50 μl of 1X Laemmli sample buffer were added to the immunocomplexes as bead pellet. The immunocomplexes were vortexed and heated to 95°C for 5 min and spinned at 10,000 x g for 5 min, and loaded onto the 10% SDS-PAGE gel for immunoblotting analysis using Cyclin B1 antibody (Santa Cruz Biotechnology).

Statistical Analysis --Each experiment was repeated at least three times. Data were expressed as mean ± SEM. Statistical comparisons were carried out by one-way analysis of variance (ANOVA). Means were examined by the Least Significant Difference post hoc analysis (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

4.4. RESULTS

Zinc supplementation markedly reduced cell growth in NHBE cells

In ZD, ZA and ZS cells, cell growth as measured by cell number per plate, was found to be reduced to 79±1.7%, 84±3.9%, and 68±5.0%, respectively, of the ZN cells **(Fig.4.1)**. The culture of cells in zinc depleted medium resulted in significant 18% reduction of cellular zinc as compared to control ZN cells **(Fig.4.2)**. Moreover, the cellular zinc level in ZA and ZS cells was 150% and 300% of that of ZN control cells. Thus, cell growth was reduced both by the low-zinc and high-zinc status, particularly in ZS cells. Furthermore, a dose-dependent elevation in cellular zinc content was observed as the zinc concentration in the media was increased.

Zinc supplementation delayed G2/M cell cycle progression in NHBE cells

To uncover the mechanism responsible for the marked cell growth reduction in ZS cells, we next examined the cell cycle progression by flow cytometry. A marked delay in G2/M cell cycle progression was observed in ZS NHBE cells (24.39±0.53%) when compared to ZN cells (13.15±0.12%) **(Fig.4.3)**. The delay was smaller in magnitude in ZA cells, with 15.59±0.20% of cells in G2/M **(Fig.4.3)**. In contrast, no change was observed in ZD cells, with 12.98±0.60% cells in G2/M, when compared to ZN cells **(Fig.4.3)**.

p53 mRNA abundance was up-regulated in ZS NHBE cells

p53 mRNA abundance was up-regulated in ZA and ZS NHBE cells to 129±2% and 196±16%, respectively, of ZN control cells (100±5%) **(Fig.4.4)**. The mRNA abundance

in ZD cells was only 17±4% of the ZN cells. Thus, in ZA and ZS cells, the marked elevations in *p53* mRNA levels were associated with increases in cellular zinc levels and reduction in cell growth. Also, the *p53* mRNA level in ZD was associated with decrease in cellular zinc levels and reduction in cell growth.

p53 protein level was markedly elevated in ZS NHBE cells

In ZS cells, western blot analysis indicated approximately a 4-fold increase in cytoplamic and a 3-fold increase in nuclear p53 protein levels when compared with the ZN cells **(Fig. 4.5A & 4.B)**. The cytoplasmic and nuclear p53 protein levels were increased 3-fold and 2.4-fold, respectively, in ZD cells; as well as 2.9-fold and 2.8-fold, respectively, in ZA cells, as compared to ZN control cells. These findings indicated that the magnitude of increases in p53 protein among the treatment groups were higher than the increases in mRNA levels, especially for the ZS group. Thus, there may be additional posttranscriptional regulation involved in the enhancement of the protein stability of p53. Because the depression of cell growth induced by an upregulation of p53 protein is well established, the marked elevation in p53 protein in ZS cells may contribute to the observed reduction in cell growth.

The blockage in G2/M cell cycle progression was alleviated by Pifithrin treatment in ZS NHBE cells

The blockage in G2/M cell cycle progression was alleviated in the ZS-Pifithrin group. Similarly, ZD-Pifithrin and ZN-Pifithrin groups showed the similar amount of cells in G2/M after inhibition of p53 transactivation by Pifithrin **(Fig.4.6)**. After

administration of Pifithrin, the cells in G2/M dropped from 8.91±0.12% to 7.42±0.27% in ZD cells, 10.76±0.98% to 8.82±1.03% in ZN cells, and 15.03±0.19% to 8.56±1.04% in ZS cells.

Gadd45 protein expression was reduced after Pifithrin treatment

The approach of suppressing p53 transactivation activity was used to establish whether the upregulated of Gadd45 was associated with elevated p53. Our data showed that the marked elevation of Gadd45 in ZS cells was reduced after the usage of Pifithrin. After inhibition of p53 transactivation activity by Pifithrin, the Gadd45 protein levels were reduced to similar levels in all Pifithrin treated cells, ZD-Pifithrin (87±8%), ZN-Pifithrin (90±3%) and ZS-Pifithrin (92±2%), as compared to ZN-controls (100±17%) **(Fig.4.7)**.

Gadd45 promoter activity was decreased after Pifithrin treatment

The approach of suppressing p53 was used to establish whether the upregulated of Gadd45 promoter activity was associated with elevated p53. Our data showed that the marked elevation of Gadd45 promoter activity in ZS cells was decreased by Pifithrin treatment. After the suppression of p53 transactivation with the Pifithrin inhibitor, the Gadd45 protein levels were reduced to similar levels in all Pifithrin treated cells, ZD-Pifithrin (87±8%), ZN-Pifithrin (90±3%) and ZS-Pifithrin (92±2%), as compared to ZN-C controls (100±17%) **(Fig.4.8)**.

Reduction in CDK1/Cyclin B1 complex level in ZS NHBE cells was normalized by p53 transactivation inhibitor Pifithrin treatment

The formation of CDK1/Cyclin B1 complex in ZS NHBE cells was lower compared to ZN control cells **(Fig. 4.9)**: ZS-control (62±4%) and ZS-mock (56±4%), as compared with ZN-control (100±17%) (Fig.8). In ZD cells, the CDK1/Cyclin B1 complex showed no significant changes, ZD-control (110±6%) and ZD-mock (113±7%), as compared with ZN-control (100±6%) **(Fig.4.9)**. To examine whether this reduction of the CDK1 and Cyclin B1 complex formation was dependent on p53 in ZS NHBE cells, suppression of p53 transactivation by inhibitor Pifithrin was performed. The amount of CDK1/Cyclin B1 complex were quantified by immunoprecipitation in all zinc treated groups, and the results showed that the depressed CDK1/Cyclin B1 complexes were normalized after suppression of p53 transactivation: ZD-Pifithrin (113±9%), ZN-Pifithrin (129±0%) and ZS-Pifithrin (108±5%), as compared to ZN-control (100±17%).

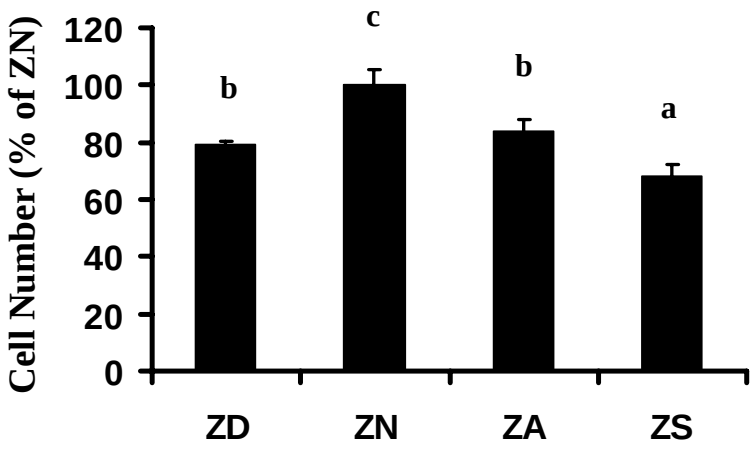


Fig. 4.1. Reduction in cell number in zinc deficient and supplemented cells.

Cell number per plate in normal human bronchial epithelial (NHBE) cells after cultured in zinc-deficient (ZD, <0.4 μM zinc), zinc-normal (ZN, 4.0 μM zinc), zinc-adequate (ZA, 16.0 μM zinc), and zinc-supplemented (ZS, 32.0 μM zinc) media for one passage. Cell number was determined by using hemacytometer. Similar results were obtained from experiments with cells from another subject.

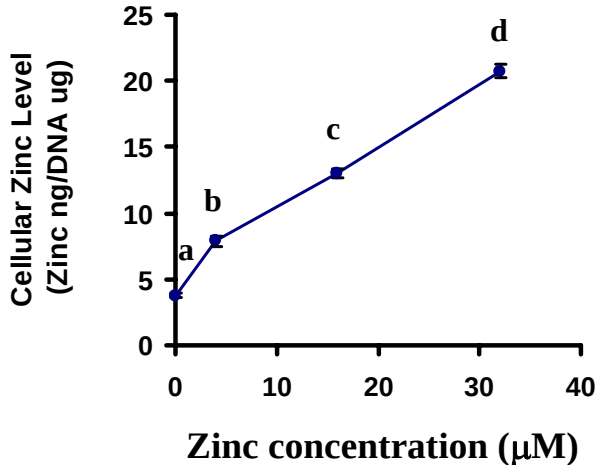


Fig. 4.2. Cellular zinc level in NHBE cells.

Cellular zinc level in NHBE cells treated with different concentrations of zinc (ZD, ZN, ZA and ZS) for one passage. Cellular zinc was measured by flame atomic absorption spectrophotometry. DNA content per plate in NHBE cells was determined by diphenylamine method. Values are means ± SEM from three experiments. Means with different letters are significantly different ($p < 0.05$). Similar results were obtained from experiments with cells from another subject.

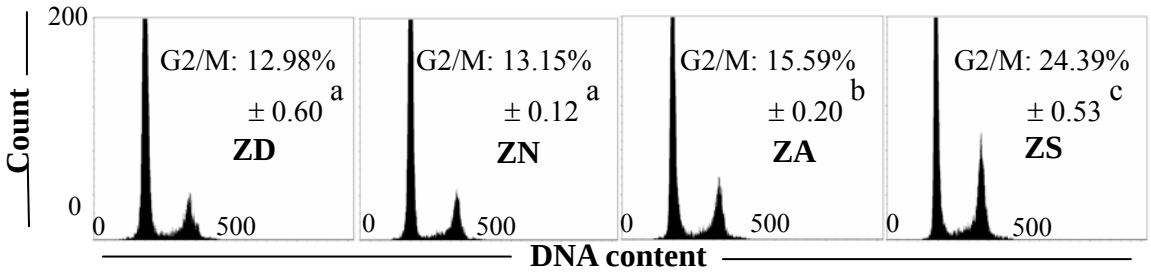


Fig. 4.3. Zinc supplementation blocked G2/M progression in NHBE cells.

Cell cycle analysis of NHBE cells was assayed by flow cytometry using a FACScalibur cytometer. Cells were cultured in ZD, ZN, ZA, and ZS media for one passage. Washed cells were fixed in ethanol and stained with propidium iodide for DNA content. Flow cytometric data files were collected and analyzed using the CELLQuest program. Cell cycle distribution percentages of stained nuclei were calculated using Modfit LT software. The calibration standard LinearFlow green and the DNA QC particle kit were used for verification of instrument performance. Histograms are representative of three independent experiments. The proportions of cells in G0/G1, S phase and the G2/M ratios are indicated for each treatment group. Similar results were obtained from experiments with cells from another subject.

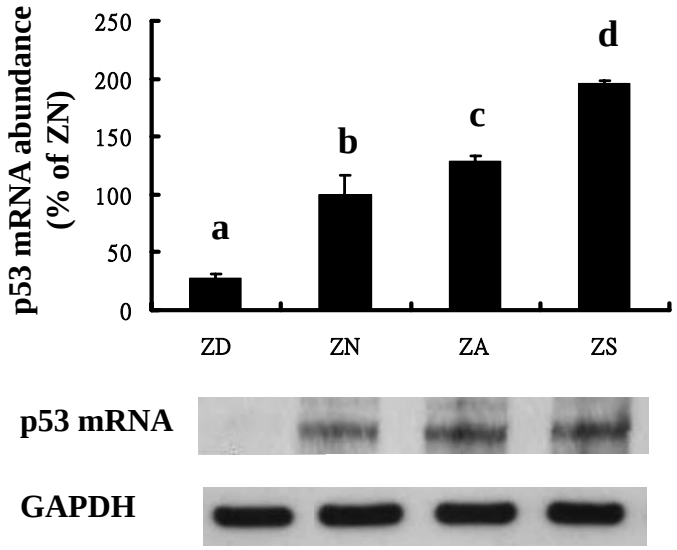


Fig. 4.4. p53 mRNA abundance was up-regulated in ZS NHBE cells.

Cells were cultured in ZD, ZN, ZA, and ZS media. Cells were cultured for one passage in BEGM with zinc added as a supplement to the ZD medium. p53 mRNA level was measured by RNase Protection Assay (RPA). RNase protection products were separated on a polyacrylamide gel and quantitated by laser densitometry. GAPDH was used as an internal reference, and values are expressed as a percentage of ZN controls. Representative samples from each treatment group are shown below the bar-graph. Values are means ± SEM from three experiments. Different letters indicate significantly different means, $p < 0.05$. Treatments with the same letters indicate no significant difference. Similar results were obtained from experiments with cells from another subject.

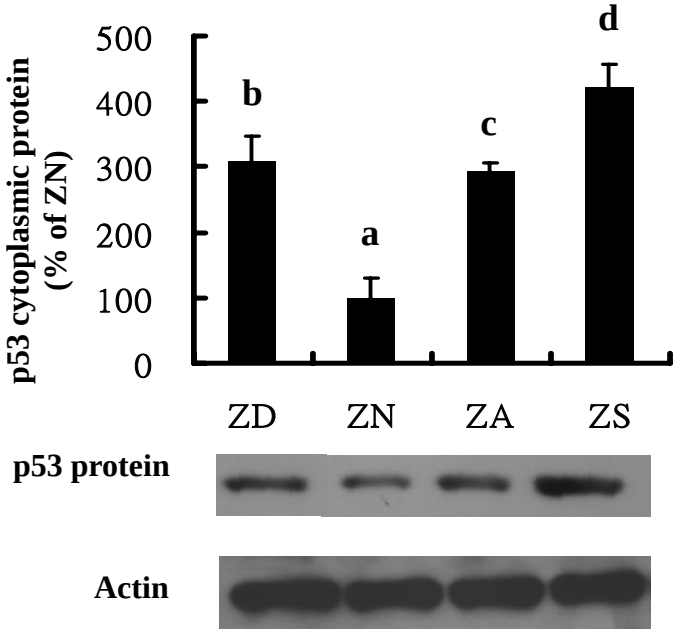


Fig. 4.5A. p53 cytoplasmic protein level was markedly elevated in ZS NHBE cells.

Cells were cultured for one passage in BEGM with zinc added as a supplement to the ZD medium. Nuclear and cytoplasmic protein extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and incubated with anti-p53 antibody. Cytoplasmic samples were probed with antibodies against Actin for normalization. Autoradiography was visualized using enhanced chemiluminescence and quantitated by densitometry. Values are expressed as a percentage of ZN controls. Representative samples, from each treatment group, are shown below the bar graph. Values are means ± SEM from 3 separated experiments. Different letters indicate significantly different means, $p < 0.05$. Similar results were obtained from experiments with cells from another subject.

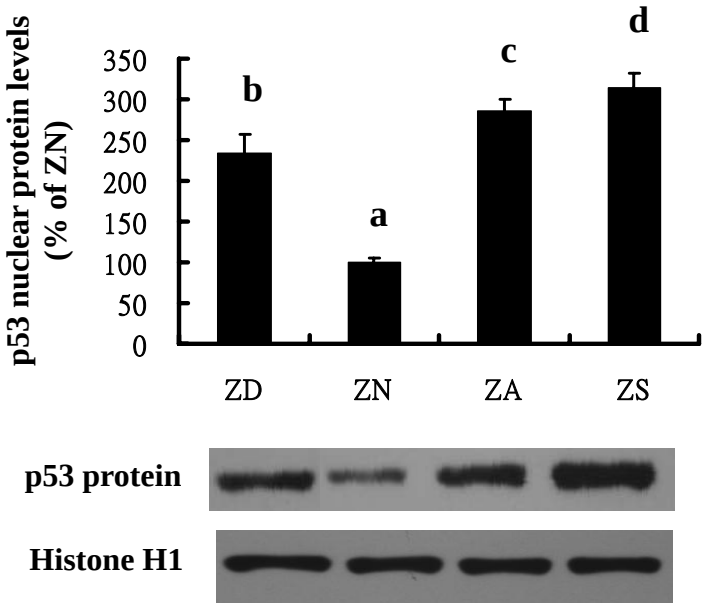


Fig. 4.5B. p53 nuclear protein level was markedly elevated in ZS NHBE cells.

Cells were cultured for one passage in BEGM with zinc added as a supplement to the ZD medium. Nuclear and cytoplasmic protein extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and incubated with anti-p53 antibody. Nuclear samples were probed with antibodies against Histone H1 for normalization. Autoradiography was visualized using enhanced chemiluminescence and quantitated by densitometry. Values are expressed as a percentage of ZN controls. Representative samples, from each treatment group, are shown below the bar graph. Values are means ± SEM from 3 separated experiments. Different letters indicate significantly different means, $p < 0.05$. Similar results were obtained from experiments with cells from another subject.

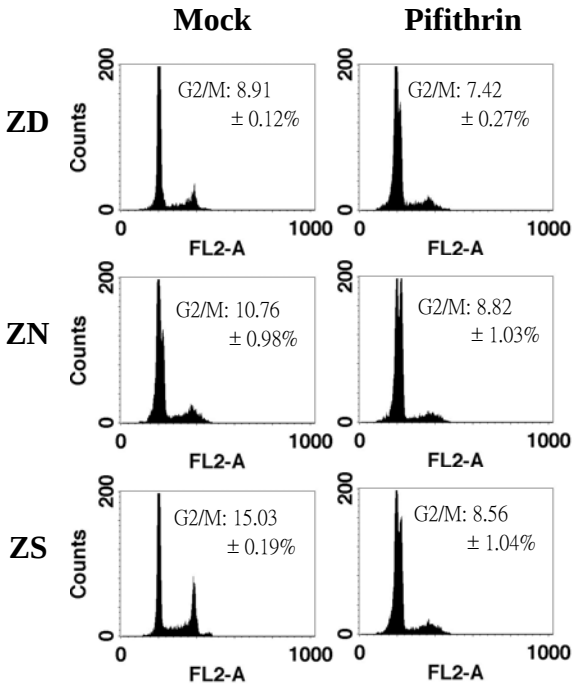


Fig. 4.6. p53 transactivation inhibitor Pifithrin treatment alleviated the blockage in G2/M.

Cell Cycle analysis after the usage of p53 transactivation inhibitor Pifithrin. Pifithrin were administrated into the medium in 5 μM for 48 hrs. Cell cycle analysis of NHBE cells was assayed by flow cytometry using a FACScalibur cytometer. Cells were fixed in ethanol and stained with propidium iodide for DNA content. Flow cytometric data files were collected and analyzed using the CELLQuestPro program. Cell cycle distribution percentages of stained nuclei were calculated using Modfit LT software. Histograms are representative of three independent experiments. Similar results were obtained from experiments with cells from another subject.

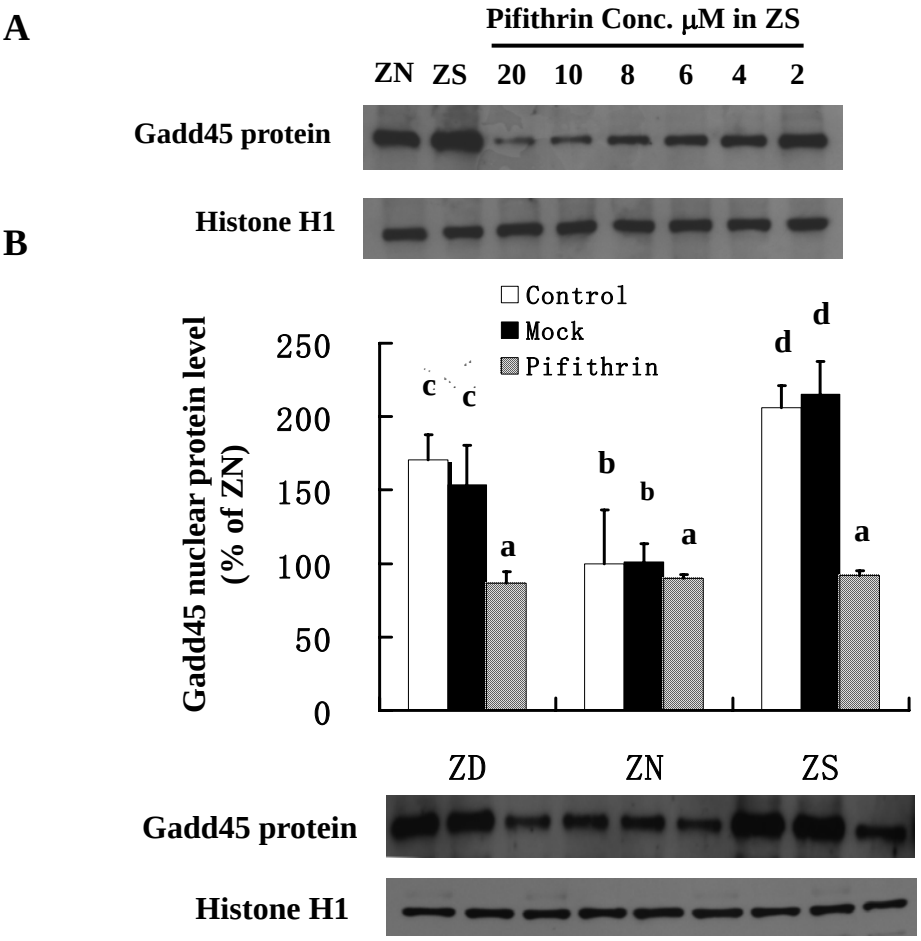


Fig.4.7. Upregulated Gadd45 protein was normalized by p53 transactivation inhibitor Pifithrin treatment. A). Gadd45 protein level after cells were treated in graded amount of Pifithrin. B). Relative nuclear Gadd45 protein levels in NHBE cells. Pifithrin were administrated into the medium in 2 μM for 48 h. Nuclear protein extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and incubated with anti-Gadd45 antibody. Nuclear samples were probed with antibodies against Histone H1 for normalization. Values are expressed as a percentage of ZN controls. Representative samples, from each treatment group, are shown below the bar graph. Values are means ± SEM from 3 separated experiments. Different letters indicate significantly different means, $p < 0.05$.

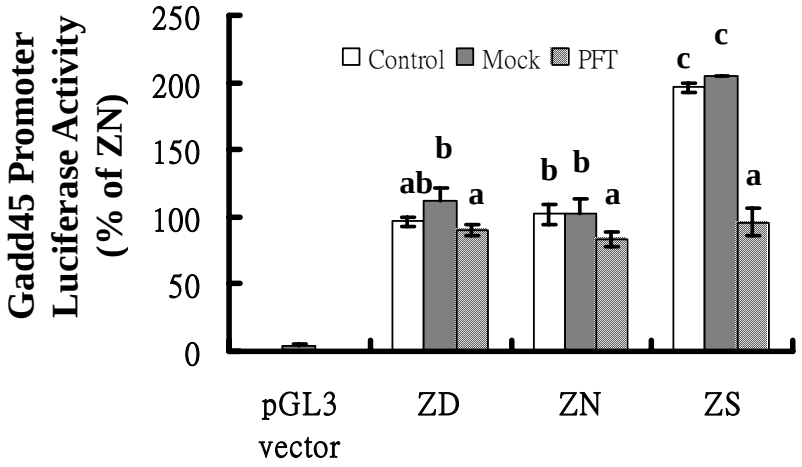


Fig. 4.8. Enhanced Gadd45 promoter activity in ZS cells was normalized by the p53 transactivation inhibitor Pifithrin.

NHBE cells were cultured in media with respective zinc concentration (ZD <0.4 μM; ZN = 4 μM; ZA = 16 μM; and ZS = 32 μM zinc supplemented to the basal medium) for 4 days. Pifithrin were administrated into the medium in 2 μM for 48 h. Meanwhile, cells were transiently transfected in serum free NHBE media by using Tfx-20 with 500 ng of luciferase reporter construct (pGL3-Gadd45) containing the Gadd45 promoter, together with 10 ng pRL-SV40 as internal control. pGL3 basal vector without carrying any promoter was used as the vector control. After transfection, the cells were cultured in corresponding media for 2 more days. Cells extracts were then assayed by Dual-Luciferase reporter system and signals were measured by a Luminometer. Values represent means ± SEM from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means ($P < 0.05$); while means with the same letter indicates no significant difference.

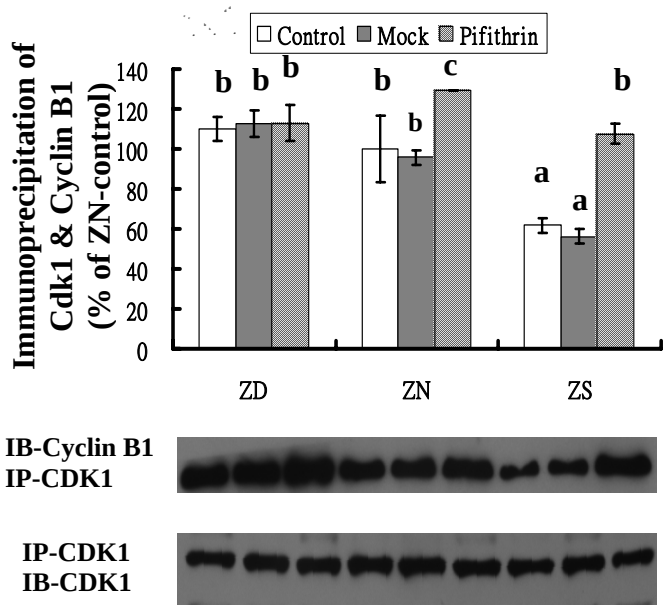


Fig. 4.9. Reduction of the CDK1 and Cyclin B1 complex level in ZS NHBE cells was normalized by the p53 transactivation inhibitor Pifithrin.

NHBE cells were cultured in media with respective zinc concentration (ZD <0.4 μM; ZN = 4 μM; ZA = 16 μM; and ZS = 32 μM zinc supplemented to the basal medium) for 4 days. Pifithrin were administrated into the medium in 2 μM for 48 h. Five hundred of proteins from whole cell lysate were added to Protein G. The complex was then centrifuged and 10 μg of CDK1 antibody or rabbit polyclonal antibody were added to the precleared lysate. Protein A/G PLUS agarose was added and centrifuged. The 50 μl of 1X Laemmli sample buffer were added to the immunocomplexes as bead pellet. The immunocomplexes were vortexed, heated, and loaded onto the 10% SDS-PAGE gel for immunoblotting analysis using Cyclin B1 antibody.

4.5. DISCUSSION

The p53 pathway is composed of a network of genes and their products that are targeted to respond to stress signals that impact upon cellular homeostatic mechanisms, which monitor DNA replication, chromosome segregation and cell division (Volgelstein et al., 2000). In response to a stress signal, the p53 protein is activated in a specific manner and this leads to either cell cycle arrest, or cellular apoptosis (Jin and Levine, 2001). The process of cell division is highly ordered and regulated. Checkpoints exist to delay progression into the next cell cycle phase only when the previous step is fully completed (Hartwell et al., 1989). Checkpoints can become activated due to DNA damage, exogenous stress signals, defects during the replication of DNA, or failure of chromosomes to attach to the mitotic spindle. Abrogation of cell cycle checkpoints can result in death for a unicellular organism or uncontrolled proliferation and tumorigenesis in metazoans (Nyberg et al., 2002). Receptor proteins can detect DNA damage and initiate signal transducers, mediators, and effector proteins to phosphorylate targets that include p53, which that eventually results in cell cycle arrest at the G1/S, intra-S, or G2/M checkpoint until the lesion undergoes repair.

G2/M progression is triggered by the maturation-promoting factor MPF, which is a complex of CDK1/Cyclin B1. p53 has been established to regulate G2/M checkpoint by transactivation of its target genes, including Gadd45. Gadd45 has been shown to interact with PCNA (Smith et al., 1994; Kearsey et al., 1995). Gadd45 also interacts with CDK1 and inhibits its kinase activity by causing reduction of the Cyclin B1/CDK1 complex formation (Zhan et al., 1999). In addition, the induction of Gadd45, which resulted in the G2 arrest is associated with translocation of cytoplasmic Cyclin B1 (Jin et al., 2002).

Moreover, the overexpression of Cyclin B1 and CDC25 abrogated the Gadd45-induced G2/M arrest. Furthermore, the induction of G2/M arrest by Gadd45 requires p53 and depends on different type of DNA damage. Human cells with downregulated Gadd45 expression or lymphocytes from Gadd45 knock-out mice failed to arrest at G2/M following UV or radiomimetic MMS, but demonstrated G2/M arrest after ionizing radiation (Wang et al., 1999).

Zinc supplementation inhibits normal human bronchial cells proliferation

Our studies were designed to investigate the influence of zinc depletion and supplementation on the cell signaling and cell cycle regulation in normal human bronchial epithelial cells (NHBE). Our findings demonstrated for the first time that the cell growth was inhibited at optimal zinc concentration previously established to be attainable in the plasma by oral supplementation. In zinc supplemented NHBE cells, cellular zinc was threefold higher than that of ZN control group **(Fig.4.2)**, and cell growth as measured by cell number was decreased to 60% of ZN **(Fig.4.1)**. Our study is the first to demonstrate that zinc supplementation, within the physiological range, is cytotoxic and reduces the growth of NHBE in primary culture. Thus, the high physiologic level of zinc also imposes adverse effect in normal cells. Controversy towards the efficacy of zinc supplementation in the prevention of cancer growth has been reported. Several studies have indicated that high cellular zinc levels inhibit cancer cell growth (Bataineh et al., 2002; Feng et al., 2000; Uzzo et al, 2002). Moreover, studies also reported that treatment with high level of zinc not only inhibited cancer cell proliferation but also induced G2/M arrest (Jaiswal et al., 2004; Liang et al., 1999). Yet, an epidemiological study has reported

an increase in risk for cancer development with usage of high level of zinc supplement (Leitzmann et al., 2003). Even though zinc supplementation can inhibit cancer cell proliferation, the cytotoxic effect of supraphysiologic zinc levels (90 and 900 μM) resulting in the induction of DNA fragmentation has been observed in human primary liver cells (Paramannantham et al., 1996).

G2/M blockage was found in ZS NHBE cells

In our studies, cell cycle analysis by flow cytometry showed there was G2/M blockage in ZS cells **(Fig. 4.3)**. In ZS cells, G2/M blockage was accompanied by three to fourfold induction of nuclear and cytoplasmic p53 proteins levels, respectively **(Fig 4.5A & 4.5B)**, and by twofold increase in mRNA expression **(Fig.4.4)** as compared to ZN control cells. These results suggest that zinc supplementation may induce cellular stress, resulted in the elevation of p53 expression. With the increase in p53 expression in ZS cells, G2/M blockage occurs to delay the transition and hinder cell growth. The underlying mechanism by which p53 blocks the G2/M cell cycle progression needs to be further investigated. This probably is due to the downstream p53 regulator, Gadd45, binding to CDK1, which decreases CDK1 kinase activity, and hinders the cell progression from G2 to M (Zhan et al., 1999). High dosage of zinc in supraphysiologic levels has been reported to induce DNA fragmentation in human cells, implying that DNA damage may occur in high zinc supplementation. The induction of G2/M arrest mediated by Gadd45 in p53-dependent and independent pathways, which depressed CDK1 kinase activity and altered subcellular Cyclin B1 levels, have been observed in many cell types when exposed to stresses (Wang et al., 1999; Mullan et al., 2001; Maeda

et al., 2002; Jiang et al., 2003; Mak et al., 2004). The effect of Gadd45 on the G2/M transition may be due to its ability to reduce complex formation of Cyclin B1and CDK1 as well as inhibit the activity of CDK1/Cyclin B1 in vitro (Zhan et al., 1999; Jin et al., 2000). In the present study, no G1 arrest was observed. This is consistent with data from another study which showed that microinjected Gadd45 did not efficiently inhibit CDKk2/Cyclin E nor caused G1 arrest (Zhan et al., 1999).

Upregulation of p53 is associated with cell growth suppression and G2/M blockage in ZS NHBE cells

Our results demonstrate that cell growth suppression was accompanied by marked induction of p53 expression, suggesting that p53 may be the main regulator in cell growth inhibition in zinc supplemented NHBE cells. To determine whether p53 is the single regulator of p53-dependent pathway in response to cellular stress, we administrated p53 inhibitor Pifithrin to suppress p53 transactivation activity. Our cell cycle analysis findings demonstrated that the G2/M blockage was alleviated by Pifithrin and normalized to similar levels as in all zinc treated cells (ZD-PFT, ZN-PFT, and ZS-PFT in **Fig. 4.6**), which suggested G2/M blockage is dependent on enhanced p53 expression in ZS NHBE cells.

Upregulation of Gadd45 is p53-dependent in ZS NHBE cells

To test whether Gadd45 expression was dependent on p53 pathway, we quantified the Gadd45 protein and assayed for the promoter activity after the p53 inhibitor Pifithrin treatment. Our result demonstrated that the Gadd45 expression and promoter activity

were dependent on the p53 pathway **(Fig. 4.7 & 4.8)**. This finding is consistent with the results obtained from p53 null cells which failed to arrest at G2/M. Our finding suggested that the upregulation of Gadd45 is dependent on p53 at the transcriptional and translational levels.

Enhanced p53 reduced CDK1/Cyclin B1 complex formation in ZS NHBE cells

Cell division relies on the expression of cyclins that bind and activate cyclin-dependent kinases to promote cell cycle progression to initiate mitosis. Association of Cyclin B with the Cyclin-dependent kinase CDK1 induces CDK1 kinase activity to promote progression through mitosis (Morgan, 1995). However, during a G2/M cell cycle checkpoint, Cyclin B is dissociated from CDK1, and these CDK1/Cyclin B1 complexes are thus reduced in the cells, which delay the progression through mitosis (Poon et al, 1996). To test whether p53 reduces the level of CDK1/Cyclin B1 complex, which formation and leads to G2/M delay in ZS NHBE cells, we examined the level of the CDK1/Cyclin B1 complex by immunoprecipitation. The amount of CDK1 and Cyclin B1 complex was decreased in ZS NHBE cells with the treatment of p53 inhibitor Pifithrin **(Fig. 4.9)**. Thus, our results suggest that the upregulation of p53, induced by zinc supplementation, enhanced the reduction the level of the CDK1/ Cyclin B1 complex and delayed the G2/M transition.

In summary, the present results presented above not only provide novel findings that zinc supplementation, in a physiologically relevant system, exerted adverse effect in normal cells, markedly elevated Gadd45 and p53 expressions and depressed CDK1/Cyclin B1 complex formation and inhibited G2/M progression were also observed

in NHBE cells. A better understanding of the mechanisms involved in the protection of normal cells from zinc cytotoxicity is essential for the identification of potential targets, and may contribute to the development of future therapeutic approaches for the treatment of human lung diseases associated with zinc toxicity.

CHAPTER V:

Enhanced activation of p38 MAPK upregulates Gadd45 and p53, reduces CDK1/Cyclin B1 complex formation and delays G2/M checkpoint in zinc supplemented bronchial epithelial cells

5.1. ABSTRACT

Supraphysiologic level of zinc can induce cytotoxic stress, DNA fragmentation and cell cycle arrest. Even an elevated zinc status within physiologic range, created by culturing cells at optimal zinc concentration attainable in the plasma by oral zinc supplementation, can induce a delay in G2/M cell cycle progression for Normal Human Bronchial Epithelial (NHBE) cells. The present study was designed to examine for the first time the involvement of p38 MAPK in G2/M delay in zinc supplemented (ZS) NHBE cells in primary culture, by the usage of p38 dominant negative (pcDNA-p38DN) and p38 protein inhibitor SB202190. In the zinc supplemented NHBE cells, we provide evidence to show that an activation of p38 MAPK upregulated Gadd45 expression, increased the cytoplasmic but decreased the nucleus CDC25B levels, reduced the CDK1/Cyclin B1 complex formation, enhanced the activation of phosphorylation of p53 at serine 15, and delayed G2/M cell cycle progression These findings demonstrate the importance of p38 MAPK in the regulation of G2/M cell cycle progression in response to the stress induced by high zinc via cell cycle checkpoint regulatory proteins, including Gadd45, CKD1, Cyclin B1 and CDC25B.

5.2. INTRODUCTION

Human exposures to high zinc stress may cause significant health risk (Merchant et al., 2001). In addition, zinc toxicity can cause acute respiratory tract inflammation together with bronchial hyper-responsiveness. Moreover, studies have shown that workers in mining industries had increased polymorpho-nuclear leukocytes and incidence of pulmonary inflammation (Blanc et al., 1991). Furthermore, DNA fragmentation has been observed in Chang liver cells cultured in medium with supraphysiologic level of zinc (Paramanantham et al., 1996). Here, we report that at the optimal physiologic concentration of zinc, attainable in plasma level by oral zinc supplementation is cytotoxic to the normal human bronchial epithelial cells in primary culture by inducing G2/M checkpoint delay. The mechanism responsible for the delay in G2/M is not fully established.

The p38 mitogen-activated protein kinase (MAPK) signaling pathway has a significant role in cell cycle regulation, apoptosis, cell development and cell differentiation (Ambrosino et al., 2001; Harper et al., 2001; Rincon et al., 2000), which are mediated by intracellular signaling pathway induced by external stimuli (UV, Ionizing Radiation), growth factors, and cytokines. In response to DNA damage, p38 MAPK is also involved in the regulation of growth-stimulation cell cycle regulators Cyclin D1, CDC25B and CDC25A (Bulavin et al., 2001).

Growth Arrest and DNA Damage inducible gene 45 (Gadd45) has been implicated in cell cycle regulation, particularly in G2/M checkpoint, in response to DNA damage. Gadd45 proteins can bind the autoinhibitory domain of MEKK4 (MKKK), which is an upstream activator of p38 MAPK, and relieve the autoinhibition of MEKK4, leading to

activation of the MAPK cascade (Mita et al., 2002). Under UV radiation stress, the induction of Gadd45 requires the activation of MAPKs (Tong et al., 2001).

p38 MAPK has been established to contribute to the activation of p53 (Bulavin et al., 1999; Bulavin et al., 2002; She et al., 2000; She et al., 2001; Renchez-Prieto et al., 2000). p53 has also been implicated in the control of the G2/M checkpoint. Introduction of wild-type p53 into p53-deficient human fibroblasts results in G1/S and G2/M arrest (Agarwal et al., 1995; Stewart et al., 1995). Evidence indicates that p53 and p21 are required for maintaining the G2 checkpoint in human HCT116 cells (Bunz et al., 1998). In addition, 14-3-3, which blocks Cdc25 activity and arrests cells at the G2/M transition, has been shown to be one of the p53 downstream genes (Hermeking et al., 1997). Gadd45 is known to play an important role in the G2/M checkpoint in response to certain types of DNA damaging agents through p53-dependent or p53-independent pathway (Jin et al., 2000; Wang et al., 1999; Zhan et al., 1999).

The present study was designed to examine for the first time the involvement of the p38 mediating signaling pathway in the delay of G2/M progression in zinc supplemented normal human epithelial cells in primary culture. Herein, we provide evidence indicating that the activation of p38 MAPK upregulated Gadd45, enhanced the activation of p53 by phosphorylating p53 at serine 15, reduced the formation of the growth-stimulating cell cycle protein CDK1/Cyclin B1 complex, reduced the nuclear by increased the cytoplasmic phosphatase CDC25B, and delayed G2/M progression in zinc supplemented normal human bronchial cells.

5.3. MATERIALS AND METHODS

Cell Culture and Treatment --NHBE cells were purchased from Cambrex Bio Science (Walkersville, MD). Cells were cultured in 100mm petri dishes containing bronchial epithelial cell growth medium (BEGM), supplemented with 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 52 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 0.5 pg/ml human recombinant epidermal growth factor, and 6.5 ng/ml triiodothyronine without antibiotics, and cultured at 37°C in a 5% CO_2 incubator. The cells were grown to 80% confluence for 6 days, and subcultured using trypsin-EDTA.

A zinc-free Bronchial Epithelial Basal Media (BEGM) baseline media, in which Cambrex omitted the addition of $ZnSO_4$, was used as the zinc-depleted media. This BEBM was supplemented with growth components, and contained residual amounts of zinc (<0.4 μM), as detected by flame atomic absorption spectrophotometry. The zinc-free basal medium of <0.4 μM was used as the zinc-depleted medium (ZD). For the other four treatment groups, zinc was added to the media in the form of $ZnSO_4$. For the zinc-normal medium (ZN) contained 4 μM of $ZnSO_4$, the zinc-adequate medium (ZA) contained 16 μM of $ZnSO_4$, and the zinc-supplemented medium (ZS) contained 32 μM $ZnSO_4$. The ZN medium was used as a comparison to standard culture media and was used as the control group for experiments. The ZA treatment was used as a representative of human plasma zinc levels, and the ZS group was used to represent the highest plasma zinc level attainable by oral supplementation in humans. After NHBE cells were subcultured into one of the four corresponding groups, the cells were cultured overnight in ZN media before changing to their respective media. The p38 inhibitor

SB202190 was purchased from Sigma-Aldrich (St. Louise, MO). Chemicals were used as stock solutions in dimethylsulfoxide. SB202190 was administrated into the cells in the working concentration of 10 μM for 48 h.

Plasmid Transfection -- NHBE cells were transiently transfected with 2 μg of dominant negative plasmid construct that was p38 MAPK cloned into pcDNA3.1 vector, by using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. The dominant negative p38 plasmid construct was a kind gift from Dr. Jiahuai Han (Zhan et al., 1999).

p38 Kinase Assay -- Kinase assay for p38 was performed with a p38 MAP kinase assay kit (cat# 9820, Cell Signaling, Danvers, CA) according to manufacturer's protocol using recombinant ATF-2 as the substrate. Activation of p38 MAPK was determined by using i) phospho-specific antibodies and Western blot analysis and (ii) a p38 MAPK assay kit.

RNase Protection Assay -- Total RNA was isolated from NHBE cells by using the RNAqueous Kit (Qiagen, Valencia, CA), according to manufacturer's instruction. The integrity of the RNA was verified by electrophoresis and quantified by spectrophotometry. The mRNA abundance of human genes including, PARP, NF-κB, TNF-β, p53, Gadd45, p38, p21, Brca1, PCNA, MDM2, were measured by non-radioactive RNase Protection Assay (Pharmingen, San Diego, CA). The human GAPDH probe was also included in the multi-probe and was used as the house-keeping gene for normalization. Labeled riboprobes were synthesized using the Non-Radioactive In Vitro Transcription kit with T7

RNA polymerase (Pharmingen), and Biotin-dUTP (Roche, Alameda, CA).

RNase Protection Assay (RPA) was performed using the Pharmingen RPA kit. The PAGE gel resolved protected probes were transferred to nylon membrane and subjected to UV crosslinking. The biotin-labeled protected cDNA transferred to the membrane were detected by chemiluminescent signal and visualized by X-ray film exposure.

Cell Cycle Analysis -- DNA contents of cells were assayed by fluorescence-activated cell sorting (FACS). NHBE cells were cultured in ZD, ZN, ZA, and ZS media for one passage, trypsinized, washed in PBS (Ca^{2+}, Mg^{2+} free), and fixed in 70% cold ethanol. Cells were stored at 4°C. For staining, cells were collected by centrifugation, and pellets were suspended in 1.0 ml propidium iodide staining solution (50 mg per ml propidium iodide, 100 U per ml RNase in PBS), and incubated at room temperature for 1 h. Staining was quantitated with a FACSCalibur cytometer (Becton Dickinson, San Jose, CA). The cell numbers were acquired with CELLQuestPro software program (Becton Dickinson, San Jose, CA). Cell cycle distribution percentages of stained nuclei were calculated by using Modfit LT software (Verity Software House, Topsham, ME). The calibration standard LinearFlow Green and the DNA QC Particle kit, for verification of instrument performance, were purchased from Molecular Probes (Eugene, OR) and Becton Dickinson, respectively.

Nuclear and Cytoplasmic Extract Preparation -- The NE-PER Nuclear and Cytoplasmic Extraction Reagents and the Halt Protease Inhibitor Cocktail Kits (Pierce Biotechnology, Rockford, IL) were used for nuclear and cytoplasmic extracts preparation

according to the manufacturer's instructions, which are based on the method of Smirnova et al. (2000). Nuclear and cytoplasmic extracts were then stored in aliquots at -80°C. Protein concentrations were determined by using the BCA Protein Assay Reagent kit (Pierce, Rockford, FL). Contaminations of nuclear extracts by cytoplasmic proteins or contamination of cytoplasmic extracts by nuclear proteins, detected by Western blot analysis of Hsp90 or Oct-1, respectively, were routinely found to be less than 5% in our lab.

Western Blot Analysis -- Nuclear and cytoplasmic protein concentrations were determined by using the Bicin Choninic Acid (BCA) kit (Pierce). Forty µg of protein were resolved on a 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Germany) by using a mini-transfer system (Bio-Rad, Hercules, CA). Membranes were blocked with 5% nonfat dry milk in PBS-T (10 mM phosphate buffer pH7.3, 137 mM NaCl, 2.7 mM KCL, and 0.1% Tween 20) for 1 h at room temperature, prior to incubation with 1 µg/ml of primary antibody from Santa Cruz Biotechnology (Santa Cruz Biotechnology), in PBS-T containing 5% nonfat milk at 4°C overnight. Membrane was then washed three times with PBS-T and blotted with a secondary antibody conjugated with horseradish peroxidase (Santa Cruz, Santa Cruz, CA) at room temperature for 1 h, followed by three washes in PBS-T. The protein was visualized by using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). Anti-Gadd45, anti-p53, anti-Cyclin B1, anti-CDC25B, anti-actin and anti-histone H1 were purchased from Santa Cruz Biotechnology.

Immunoprecipitation of CDK1/CyclinB1 – After reaching 70% confluency, cells were harvested and washed with 10 ml of PBS. Cells were then washed and resuspended in 1 ml of cold lysis Buffer containing 1X Protease Inhibitor Cocktail (Pierce, Rockford, IL). Cells were then placed on ice for 30 min and centrifuged at 10,000 x g for 15 min at 4^{o}C. The supernatant was collected in a new microcentrifuge tube. Protein concentration in the whole cell lysates were determined by BCA reagent kit (Pierce). Five hundred μg of proteins from whole cell lysate were added to 50 μl of Protein G (Santa Cruz Biotechnology), washed with cold lysis buffer, and incubated on ice for 1 h. The complex was then centrifuged at 10,000 x g for 10 min at 4°C. Ten μg of Cyclin B1 antibody or rabbit polyclonal antibody (Santa Cruz Biotechnology) were added to the precleared lysate and incubated for 1 h. Protein A/G PLUS agarose (Santa Cruz Biotechnology) was added and incubated at 4°C overnight on a rocking platform. The immunocomplexes were centrifuged at 10,000 x g for 30 seconds at 4°C and washed five times with 500 μl of lysis buffer. The 50 μl of 1X Laemmli sample buffer were added to the immunocomplexes as bead pellet. The immunocomplexes were vortexed and heated to 95°C for 5 min and centrifuged at 10,000 x g for 5 min, and loaded onto the 10% SDS-PAGE gel for immunoblotting analysis using Cyclin B1 antibody (Santa Cruz Biotechnology).

Statistical Analysis --Each experiment was repeated at least three times. Data were expressed as means ± SEM. Statistical comparisons were carried out by one-way analysis of variance (ANOVA). Means were examined by the Least Significant Difference post hoc analysis (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

5.4. RESULTS

Zinc supplementation markedly reduced cell growth in normal human bronchial cells

In ZD, ZA and ZS cells, cell growth as measured by DNA content per plate, was found to be reduced to 83%, 79%, and 67%, respectively, of the ZN cells **(Fig.5.1A)**. The culture of cells in zinc depleted medium resulted in significant 18% reduction of cellular zinc as compared to control ZN cells **(Fig.5.1B)**. Moreover, cellular zinc level in ZA and ZS cells was 150% and 300% of that of ZN control cells. Thus cell growth was reduced both by the low-zinc and high-zinc status, particularly in ZS cells. Furthermore, a dose-dependent elevation in cellular zinc content was observed as the zinc concentration in the media was increased.

p38 mRNA abundance was up-regulated in ZS NHBE cells

p38 mRNA abundance was up-regulated in ZA and ZS NHBE cells to 195±31% and 307±24%, respectively, of ZN control cells (100±21%) **(Fig.5.1C)**. The mRNA abundance in ZD cells was 90±4% of ZN cells but the difference is not significant. Thus, in ZA and ZS cells, the marked elevations in *p38* mRNA levels were associated with increases in cellular zinc levels and reduction in cell growth.

p38 kinase activity was markedly elevated in ZS NHBE cells

p38 kinase activities were increased in ZD, ZA and ZS cells to 177±10%, 238±24% and 256±14%, respectively, to ZN (100±7%) control cells **(Fig.5.2)**. These findings indicated the phosphorylation of p38 MAPK was upregulated in ZD, ZA and ZS, with the

highest increase in ZS cells.

Suppression of p38 by Dominant Negative and protein inhibitor alleviated the delay in G2/M

To determine that the increase in p38 expression and activation is associated with the delay in G2/M in ZS cells, we knocked down p38 kinase activity by dominant negative and protein inhibitor SB202190 in NHBE cells. Cell cycle progression was analyzed by flow cytometry after the suppression of p38. After the application of p38 dominant negative, the percentage of cells in G2/M was decreased from 8.81±0% to 7.07±0.06% in ZD cells, from 9.40±0.17% to 8.67±0.05% in ZN cells, and from 14.54±0.44 to 8.98±0.72% in ZS cells, respectively **(Fig. 5.3A)**. Similarly, following the administration of p38 protein inhibitor SB202190, the percentage of cells in G2/M was decreased from 21.29±0.32% to 17.73±1.61% in ZD cells, from 20.47±0.28% to 17.70±0.84% in ZN cells, and from 25.75±0.16% to 16.24±0.54% in ZS cells, respectively **(Fig. 5.3B)**. These findings demonstrated that the G2/M delay in ZS cells was dependent on the upregulation of p38 MAPK kinase activity.

Upregulation of Gadd45 protein was normalized after suppression of p38 by dominant-negative and protein inhibitor

To determine whether the enhanced Gadd45 protein was associated with the elevated p38 MAPK activity in ZS NHBE cells, we measured the nuclear protein level of Gadd45 after suppression of p38 with dominant negative and protein inhibitor (SB202190). The Gadd45 nuclear protein levels were knocked down to similar levels in

all p38 suppression groups treated with dominant-negative p38 (pcDNA-p38DN) **(Fig.5.4A & 5.4B)**, ZD-pcDNA-p38DN (70±12%), ZN-pcDNA-p38DN (40±2%) and ZS-pcDNA-p38DN (66±13%), as compared to ZN-control (100±1%) **(Fig.5.4A)**. Similarly, Gadd45 nuclear protein showed the similar trend in suppression of p38 by protein inhibitor SB202190, in ZD-SB202190 (54±17%), ZN-SB202190 (42±11%) and ZS-SB202190 (53±16%) cells, as compared to ZN-control cells (100±28%) **(Fig.5.4B)**. These findings suggest that in ZS NHBE cells the upregulation of Gadd45 is dependent on the markedly elevated p38 MAPK expression.

Enhanced phosphorylation of p53 at serine 15 was diminished after suppression of p38 by dominant-negative p38

To test whether the enhanced phosphorylation of p53 was dependent on the elevated p38 expression in ZS NHBE cells, the amount of phosphorylated p53 (serine 15) was quantified after the administration of dominant-negative p38 among all treatments. The result showed that the enhanced phosphorylated p53 (serine 15) was diminished after suppression of p38 by dominant negative (Fig.5), in ZD-pcDNA-p38DN (22±4%), ZN-pcDNA-p38DN (38±5%) and ZS-pcDNA-p38DN (25±2%) cells, as compared to ZN-control cells (100±5%) cells. This result suggests that the enhanced phosphorylated p53 at serine 15 is dependent on p38 MAPK in ZS NHBE cells.

Formation of CDK1 and Cyclin B1 in ZS NHBE cells was normalized after suppression of p38 by dominant negative and protein inhibitor

In ZS NHBE cells, the formation of CDK1/Cyclin B1 complex was lower than ZN

control cells (Fig. 6A & 6B): ZS-control (53±7%) and ZS-pcDNA (58±3%) cells, as compared with ZN-control (100±6%) cells **(Fig.5.6A)**; ZS-control (52±5%) and ZS-Mock (64±6%) cells, as compared with ZN-control (100±17%) cells **(Fig.5.6B)**. In ZD cells, the CDK1/Cyclin B1 complex showed no significant changes: ZD-control (118±9%) and ZD-pcDNA (103±9%) cells, as compared with ZN-control (100±6%) cells **(Fig.5.6A)**; ZD-control (120±15%) and ZD-Mock (96±1%) cells, as compared with ZN-control (100±17%) cells **(Fig.5.6B)**. To examine whether this reduced formation of the complex was dependent on p38 MAPK in ZS NHBE cells, the dominant-negative p38 and protein inhibitor SB202190 were administrated to suppress p38 MAPK. In ZS cells, the reduction in CDK1/Cyclin B1 complex was normalized after suppression of p38 by dominant negative: ZD-pcDNA-p38DN (115±17%), ZN-pcDNA-p38DN (104±2%) and ZS-pcDNA-p38DN (108±9%), as compared to ZN-control (100±6%). Similarly, the reduced formation of CDK1/Cyclin B1 complex in ZS NHBE cells was normalized when p38 was suppressed by protein inhibitor SB202190: ZD-SB202190 (114±8%), ZN-SB202190 (104±1%) and ZS-SB202190 (105±5%) cells, as compared to ZN-control (100±17%) cells.

Distribution of CDC25B in cytoplasmic and nuclear compartments was affected after suppression of p38 by dominant-negative p38 and protein inhibitor

The nuclear phosphatase CDC25B protein in ZA and ZS cells were reduced to 49±4% and 30±5%, respectively, of ZN cells (100±13%) **(Fig.5.7A)**. However, the cytoplasmic phosphatase CDC25B proteins were higher in ZD-control (118±9%), ZD-pcDNA (175±29%), ZS-control (269±4%), ZS-pcDNA (234±16%), as compared to

ZN-control (100±12%) cells **(Fig. 5.7B)**. To test whether the distorted proportion of CDC25B between cytoplasm and nucleus was dependent on p38 MAPK, we used dominant-negative p38 and protein inhibitor to suppress p38. The results demonstrated that the higher amounts of CDC25B in the cytoplasm of ZD and ZS NHBE cells were normalized after the administration of dominant negative of p38. The amount of CDC25B in ZD-pcDNA-p38DN (58±26%), ZN-pcDNA-p38DN (62±26%) and ZS-pcDNA-p38DN (57±22%) cells, were not different from the ZN-control cells (100±12%) cells **(Fig.5.7B)**. Similarly, the amount of CDC25B in the cytoplasm of ZD and ZS cells treated with protein inhibitor SB202190 were decreased to similar levels: ZD-SB202190 (62±9%), ZN-SB202190 (66±7%) and ZS-SB202190 (66±19%) cells, and were not different from the ZN-control cells (100±31%).

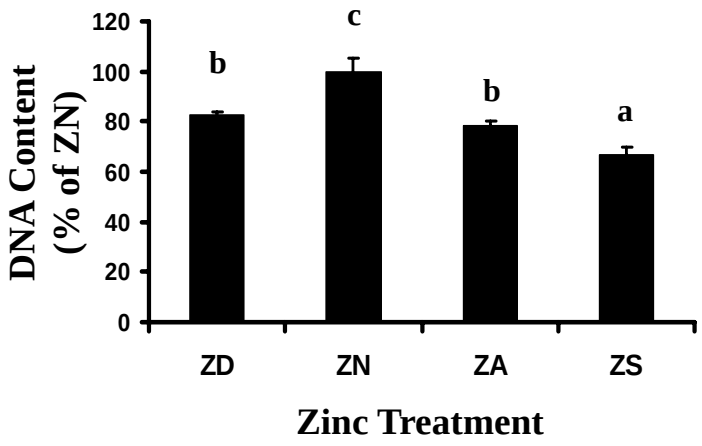


Fig. 5.1A. Zinc supplementation reduced cell growth in NHBE cells.

DNA content per plate in normal human bronchial epithelial (NHBE) cells after cultured in zinc-deficient (ZD, <0.4 μM zinc), zinc-normal (ZN, 4.0 μM zinc), zinc-adequate (ZA, 16.0 μM zinc), and zinc-supplemented (ZS, 32.0 μM zinc) media for one passage. DNA content was determined by the diphenylamine method. Values are means ± SEM from three experiments. Different letters indicate significantly different means, $p < 0.05$. Treatments with the same letters indicate no significant difference. Similar results were obtained from experiments with cells from another subject.

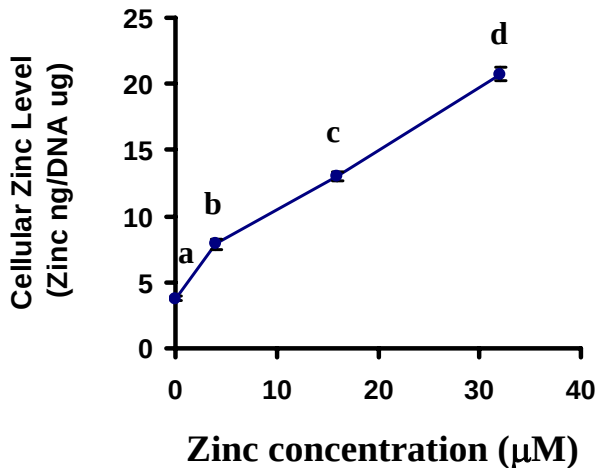


Fig. 5.1B. Cellular zinc level in NHBE cells.

Cells after cultured in zinc-deficient (ZD, <0.4 μM zinc), zinc-normal (ZN, 4.0 μM zinc), zinc-adequate (ZA, 16.0 μM zinc), and zinc-supplemented (ZS, 32.0 μM zinc) media for one passage. Cellular zinc level in NHBE cells treated with different concentrations of zinc (ZD, ZN, ZA and ZS) for one passage. Cellular zinc was measured by flame atomic absorption spectrophotometry. Values are means ± SEM from three experiments. Means with different letter are significantly different ($p < 0.05$).

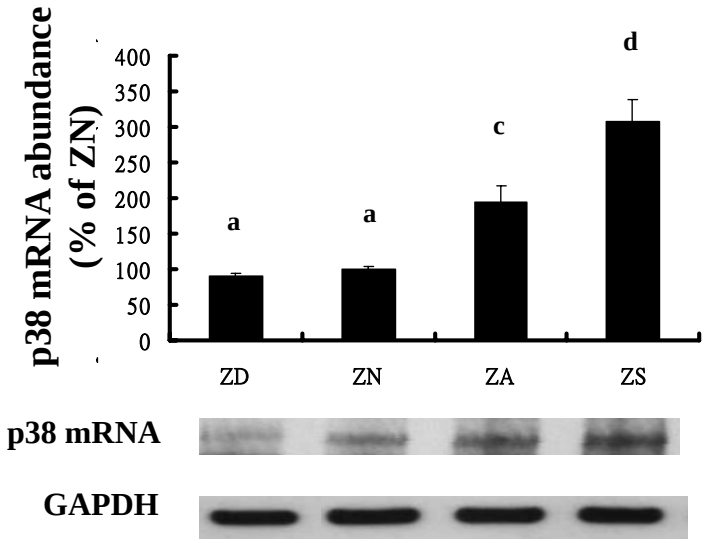


Fig. 5.1C. p38 mRNA abundance was up-regulated in ZS NHBE cells.

Cells were cultured in ZD (<0.4 μM zinc), ZN (4.0 μM zinc), ZA (16.0 μM zinc), and ZS (32.0 μM zinc) media. Cells were cultured for 1 passage in BEGM with zinc added as a supplement to the ZD medium. p38 MAPK mRNA level was measured by RNase Protection Assay (RPA). RNase protection products were separated on a polyacrylamide gel and quantitated by laser densitometry. GAPDH was used as an internal reference, and values are expressed as a percentage of ZN controls. Representative samples from each treatment group are shown below the bar-graph. Values are means ± SEM from three experiments. Different letters indicate significantly different means, $p < 0.05$. Treatments with the same letters indicate no significant difference.

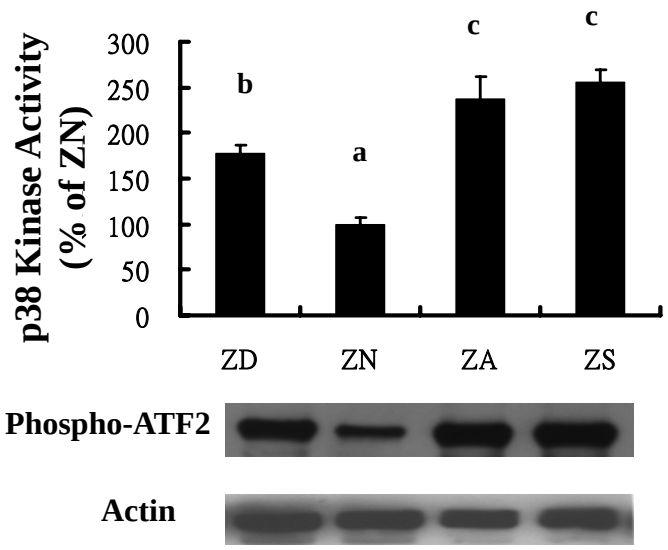


Fig. 5.2. p38 kinase activity was markedly elevated in ZS NHBE cells.

Cells were cultured in ZD (<0.4 μM zinc), ZN (4.0 μM zinc), ZA (16.0 μM zinc), and ZS (32.0 μM zinc) media. Cells were cultured for 1 passage in BEGM with zinc added as a supplement to the ZD medium. Kinase assay for p38 was performed with a p38 MAP kinase assay kit (Cell Signaling Inc.) according to manufacturer's protocol by using recombinant ATF-2 as substrate. Activation of p38 MAPK was determined by using i) phospho-specific antibodies and Western blot analysis and (ii) a p38 MAPK assay kit. Representative samples from each treatment group are shown below the bar-graph. Values are means ± SEM from three experiments. Different letters indicate significantly different means, $p < 0.05$. Treatments with the same letters indicate no significant difference.

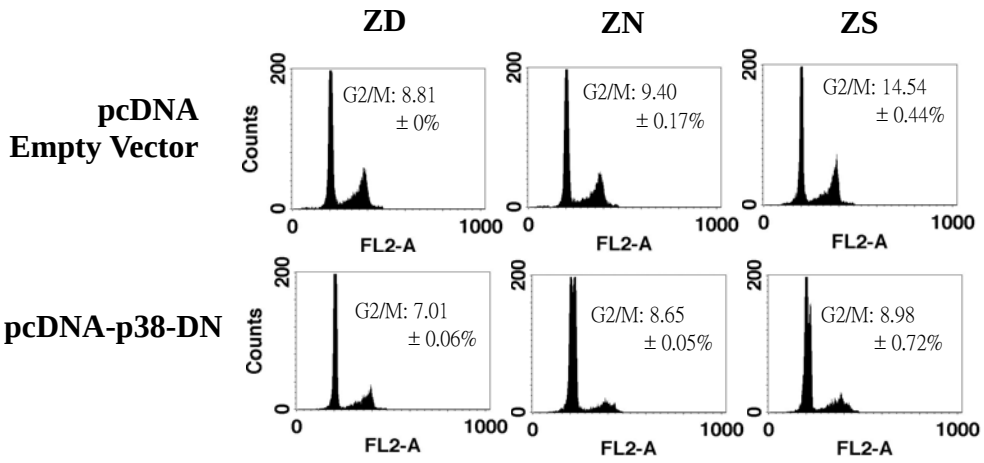


Fig. 5.3A. Cell Cycle analysis after suppression of p38by dominant negative and alleviated the delay in G2/M. Cell Cycle analysis after suppression of p38 MAPK by dominant negative. Cells were transiently transfected with 2 μg of pcDNA-p38DN for 48 hrs with Lipofectamine. Cell cycle analysis of NHBE cells was assayed by flow cytometry using a FACScalibur cytometer. Cells were fixed in ethanol and stained with propidium iodide for DNA content. Flow cytometric data files were collected and analyzed using the CELLQuest program. Cell cycle distribution percentages of stained nuclei were calculated using Modfit LT software.

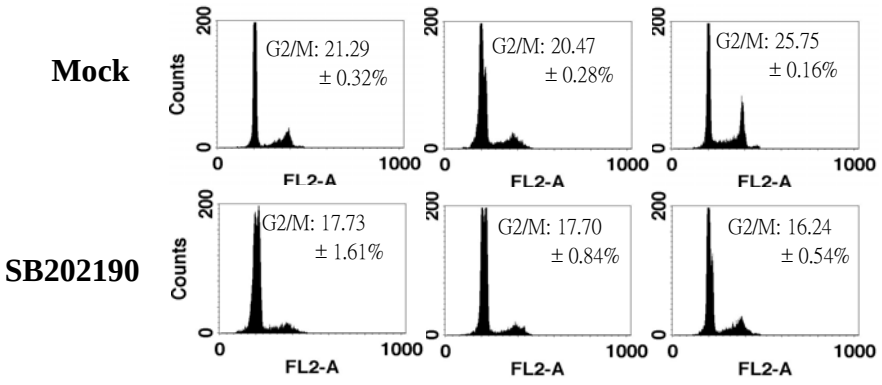


Fig. 5.3B. Cell Cycle analysis after suppression of p38 by inhibitor SB202190.

SB202190 were administrated into the medium at 10 μM for 48 h. Cell cycle analysis of NHBE cells was assayed by flow cytometry using a FACScalibur cytometer. Cells were fixed in ethanol and stained with propidium iodide for DNA content. Flow cytometric data files were collected and analyzed using the CELLQuest program. Cell cycle distribution percentages of stained nuclei were calculated using Modfit LT software.

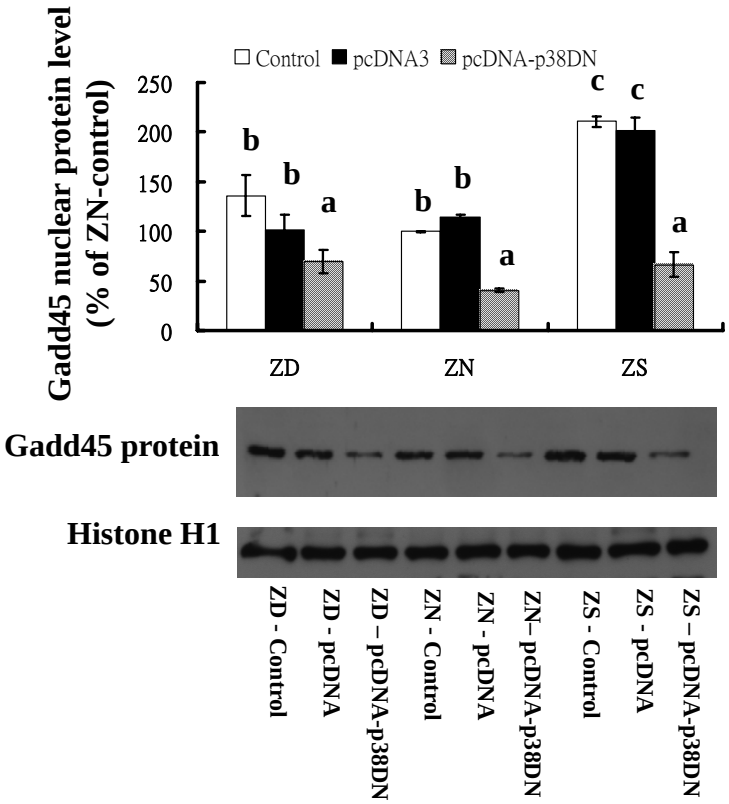


Fig. 5.4A. Enhanced Gadd45 protein expression was normalized after suppression of p38 by dominant-negative. Gadd45 protein level after suppression of p38 by dominant negative. Relative nuclear Gadd45 protein levels in NHBE cells. Nuclear protein extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and incubated with anti-Gadd45 antibody. Nuclear samples were probed with antibodies against Histone H1 for normalization. Autoradiography was visualized by using enhanced chemiluminescence and quantitated by densitometry. Values are expressed as a percentage of ZN controls. Representative samples, from each treatment group, are shown below the bar graph. Values are means ± SEM from 3 separated experiments. Different letters indicate significantly different means, $p < 0.05$.

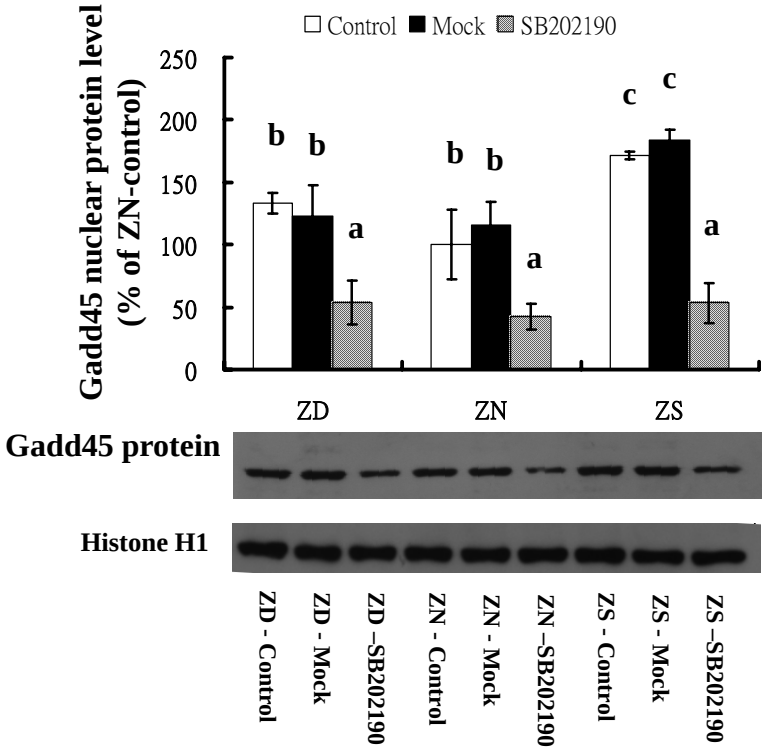


Fig.5.4B. Enhanced Gadd45 protein expression was normalized after suppression of p38 by SB202190. Gadd45 protein level after suppression of p38 by inhibitor SB202190. Relative nuclear Gadd45 protein levels in NHBE cells. Nuclear protein extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and incubated with anti-Gadd45 antibody. Nuclear samples were probed with antibodies against Histone H1 for normalization. Autoradiography was visualized by using enhanced chemiluminescence and quantitated by densitometry. Values are expressed as a percentage of ZN controls. Representative samples, from each treatment group, are shown below the bar graph. Values are means ± SEM from 3 separated experiments. Different letters indicate significantly different means, $p < 0.05$.

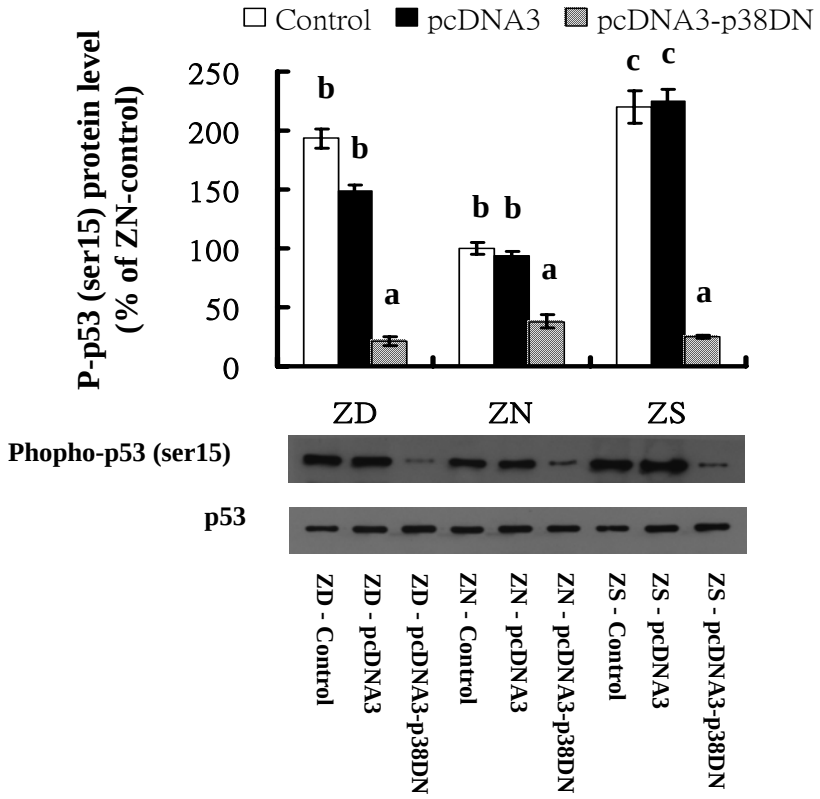


Fig. 5.5. Accumulation of phosphorylated p53 was diminished after suppression of p38 by dominant-negative p38

Total lysate extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and incubated with anti-phospho-p53 (ser15) antibody. Autoradiography was visualized by using enhanced chemiluminescence and quantitated by densitometry. Values are expressed as a percentage of ZN controls. Representative samples, from each treatment group, are shown below the bar graph. Values are means ± SEM from 3 separated experiments. Different letters indicate significantly different means, $p < 0.05$.

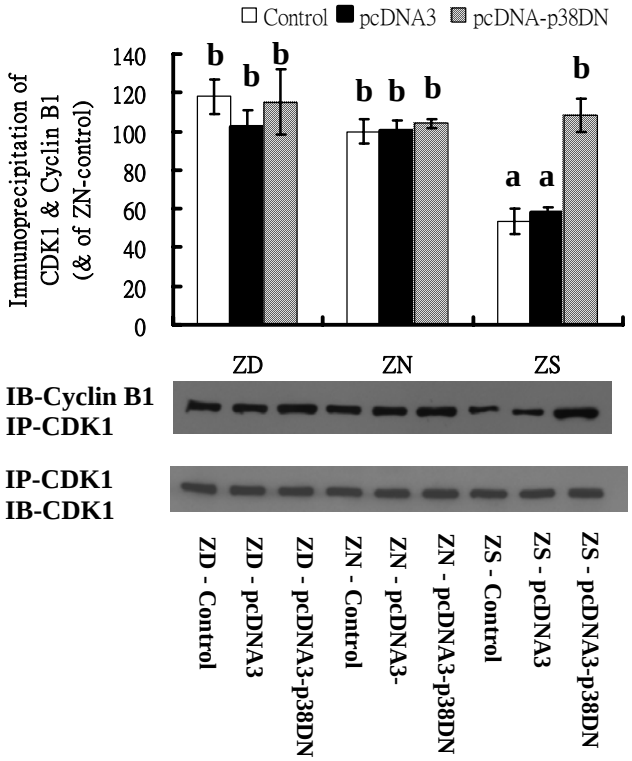


Fig. 5.6A. Reduction of CDK1/Cyclin B1 complex formation in ZS NHBE cells was normalized after suppression of p38 by dominant negative.

CDK1/Cyclin B1 complex formation after suppression of p38 by dominant negative. Five hundreds μg of proteins from whole cell lysate were added to Protein G. The complex then centrifuged and 10 μg of CDK1 antibody or rabbit polyclonal antibody was added to the precleared lysate. Protein A/G PLUS agarose was added and centrifuged. The 50 μl of 1X Laemmli sample buffer was added to the immunocomplexes as bead pellet. The immunocomplexes were vortexed, heated and loaded onto the 10% SDS-PAGE gel for immunoblotting analysis using Cyclin B1 antibody.

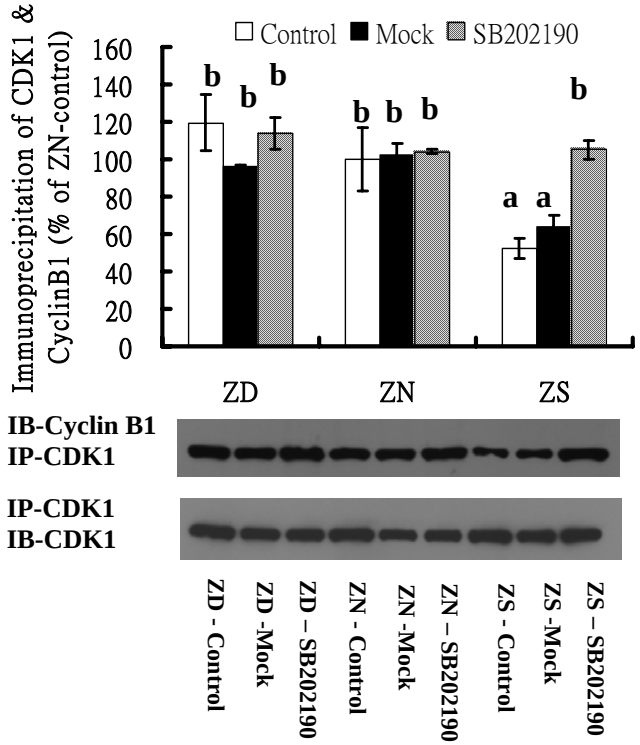


Fig. 5.6B. Reduction of CDK1/Cyclin B1 complex formation in ZS NHBE cells was normalized after suppression of p38 by protein inhibitor.

CDK1/Cyclin B1 complex formation after suppression of p38 MAPK by inhibitor SB202190. Five hundreds μg of proteins from whole cell lysate were added to Protein G. The complex then centrifuged and 10 μg of CDK1 antibody or rabbit polyclonal antibody was added to the precleared lysate. Protein A/G PLUS agarose was added and centrifuged. The 50μl of 1X Laemmli sample buffer was added to the immunocomplexes as bead pellet. The immunocomplexes were vortexed, heated and loaded onto the 10% SDS-PAGE gel for immunoblotting analysis using Cyclin B1 antibody.

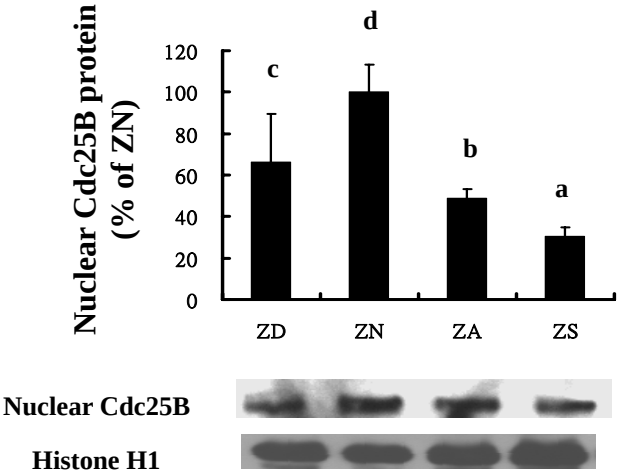


Fig. 5.7A. Enhanced shuttling of CDC25B from nucleus into cytoplasm was reduced by use of p38 dominant negative and protein inhibitor in ZS NHBE cells. Nuclear CDC25B protein level. Nuclear and cytoplasmic protein extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and incubated with anti-CDC25B antibody. Cytoplasmic and nuclear samples were probed with antibodies against Actin and Histone H1, respectively, for normalization. Autoradiography was visualized using enhanced chemiluminescence and quantitated by densitometry. Values are expressed as a percentage of ZN controls. Representative samples, from each treatment group, are shown below the bar graph. Values are means ± SEM from 3 separated experiments. Different letters indicate significantly different means, $p < 0.05$.

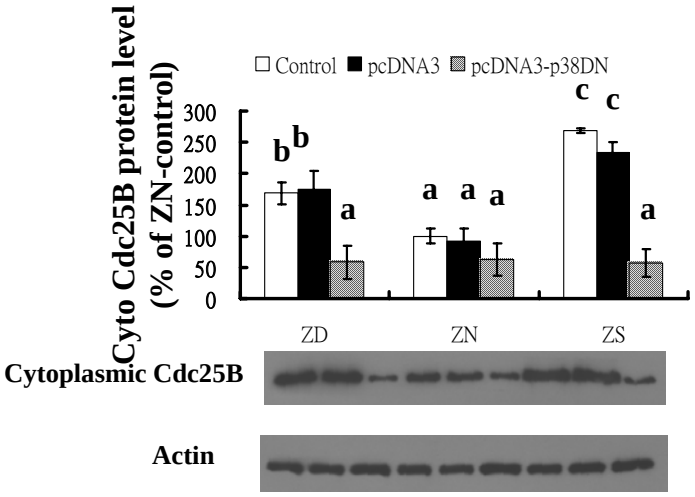


Fig. 5.7B. Enhanced shuttling of CDC25B from nucleus into cytoplasm was normalized by use of p38 dominant negative and protein inhibitor in ZS NHBE cells. Cytoplasmic CDC25B protein levels after suppression of p38 by dominant negative. Nuclear and cytoplasmic protein extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and incubated with anti-CDC25B antibody. Cytoplasmic and nuclear samples were probed with antibodies against Actin and Histone H1, respectively, for normalization. Autoradiography was visualized using enhanced chemiluminescence and quantitated by densitometry. Values are expressed as a percentage of ZN controls. Representative samples, from each treatment group, are shown below the bar graph. Values are means ± SEM from 3 separated experiments. Different letters indicate significantly different means, $p < 0.05$.

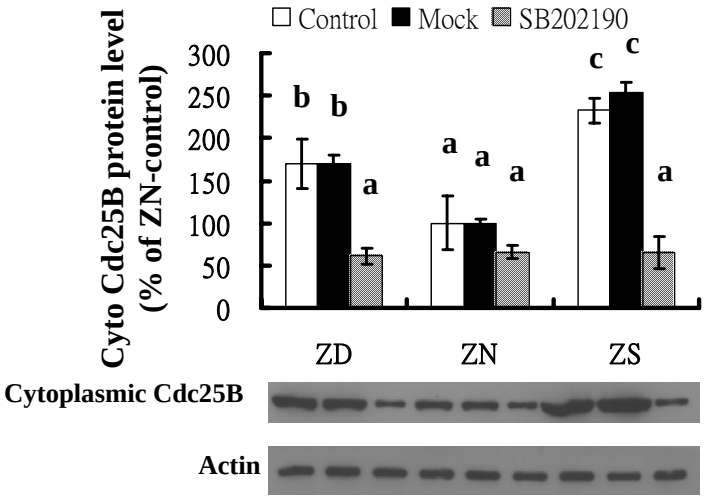


Fig. 5.7C. Enhanced shuttling of CDC25B from nucleus into cytoplasm was normalized by use of p38 dominant negative and protein inhibitor in ZS NHBE cells. Cytoplasmic CDC25B protein levels after suppression of p38 by protein inhibitor SB202190. Nuclear and cytoplasmic protein extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and incubated with anti-CDC25B antibody. Cytoplasmic and nuclear samples were probed with antibodies against Actin and Histone H1, respectively, for normalization. Autoradiography was visualized using enhanced chemiluminescence and quantitated by densitometry. Values are expressed as a percentage of ZN controls. Representative samples, from each treatment group, are shown below the bar graph. Values are means ± SEM from 3 separated experiments. Different letters indicate significantly different means, $p < 0.05$.

5.5. DISCUSSION

p38 MAPK mRNA abundance and kinase activity were enhanced in ZS NHBE cells

In this report, we present data for the first time that p38 mRNA abundance was upregulated and the kinase activity was induced in zinc supplemented NHBE cells cultured at optimal zinc concentration, which has been shown to be attainable in the plasma by oral supplementation.

Activation of p38 MAPK induced p53 phosphorylation in position serine 15

The activation of p38 MAPK has been shown to arrest G2/M progression by phosphorylation and stabilization of p53 in response to DNA damage induced by UV radiation in human fibroblasts (Bulavin et al, 1999; She et al, 2000). To test whether the elevated p38 MAPK mRNA abundance and activated p38 MAPK activity may cause the accumulation of phosphorylated p53 (ser 15) in ZD and ZS NHBE cells, we examined the phosphorylated p53 levels in ZD, ZN, and ZS NHBE cells with or without the suppression of p38 MAPK by dominant negative (pcDNA-p38DN). This approach established that the accumulation of phosphorylated p53, in ZD and ZS NHBE cells, is dependent on p38 MAPK kinase activity because treatment of these cells with p38 MAPK dominant negative resulted in a marked reduction in the phosphorylated p53 protein levels **(Fig. 5.5.)**.

Activation of p38 MAPK induced Gadd45 protein expression

The dependency of p38 in the role of Gadd45 protein in regulating cell cycle progression is a controversial topic. In medulloblastoma cells, the Gadd45 induced

apoptosis in response to nerve growth factor stimulation was found to be independent on the p38 kinase pathways (Chou et al., 2001). In contrast, in UV-irradiated skin tumor, the induction of of Gadd45 on apoptosis was dependent on p38 kinase activity (Hildesheim et al., 2002). Overexpression of Gadd45 in human fibroblasts induced neither apoptosis nor p38 kinase activity (Sheika et al., 2000). Moreover, in Gadd45-deficient mice radiation-induced apoptosis or activation of p38 kinase pathway is not inhibited (Sheika et al., 2000). Gadd45 has been established to bind to MTK1 and activate MTK1 downstream activators including p38 (Takekawa et al., 1998). In our results, we showed that the upregulation of Gadd45 protein is dependent on p38 MAPK in ZS NHBE cells. When the activated p38 MAPK was suppressed by dominant negative and inhibitor, the elevated Gadd45 protein was reduced markedly in ZD, ZN and ZS NHBE cells **(Fig. 5.4A & 5.4B)**. These findings provided evidence to support the contention that the upregulated Gadd45 is dependent on p38 MAPK.

Delay in G2/M checkpoint was dependent on p38 MAPK

Cell division relies on the expression of cyclins that bind and activate cyclin-dependent kinases to promote cell cycle progression to initiate mitosis. Association of Cyclin B1 with the Cyclin-dependent kinase CDK1 induces CDK1 kinase activity to promote progression through mitosis (Morgan, 1995). However, during the G2/M cell cycle checkpoint, Cyclin B1 dissociates from CDK1 and results in a decrease in the Cyclin B/CDK1 complex in the cells, which delays the progression through mitosis (Poon et al, 1996). To examine whether the delay in G2/M in ZS NHBE cells is dependent on p38 MAPK, we investigated the influence of p38 MAPK on the G2/M

transition by suppressing p38 MAPK with dominant negative (pcDNA-p38DN) and inhibitor SB202190. In ZS cells, the delay in G2/M was alleviated after suppression of p38 MAPK, and the amount cells in G2/M were relatively the same in all zinc treatment group **(Fig. 5.3A & 5.3B)**, suggesting that the G2/M delay in ZS is dependent on p38 MAPK. After showing evidence of the dependency of G2/M delay in p38 MAPK in ZS cells, we then examined the CDK/Cyclin B1 complex formation by immunoprecipitation. The amount of CDK1/Cyclin B1 complex was markedly reduced in ZS NHBE cells **(Fig.5.6)**, suggesting that the cellular stress induced by zinc supplementation lead to the reduction of the CDK1 and Cyclin B1 complex formation and the delay in G2/M transition. To establish whether the decrease in the amount of CDK1/CyclinB1 complex is dependent on the activation of p38 MAPK, we determined the amount of the complex after suppression of p38 MAPK by dominant negative and inhibitor. Our results demonstrated that the decreased of CDK1/Cyclin B1 complex in ZS cells was restored and normalized to the same level of ZN controls after the suppression of p38 MAPK **(Fig.5.6A & 5.6B)**, suggesting that the decreased CDK1/Cyclin B1 complex is due to the increased p38 kinase activity. These results indicate that p38 MAPK reduced the formation of the CDK1 and Cyclin B1 complex formation and delays the G2/M in ZS NHBE cells.

p38 MAPK translocated phosphatase CDC25B from the nucleus to cytoplasm and caused the delay in G2/M in ZS NHBE cells

The CDC25B phosphatase functions as essential regulators of cell cycle progression during normal eukaryotic cell division and as mediators of the checkpoint response in

cells with DNA damage (Strausfeld et al., 1991). CDC25B dephosphorylates phospho-Thr14 and phospho-Tyr15 on the CDK1/Cyclin B1 complex. Dephosphorylation of the CDK-cyclin complex leads to activation of these regulatory kinases, phosphorylation of their numerous cellular targets, and G2/M cell cycle progression (Morgan et al., 1997). The importance of CDC25B in cell cycle regulation is emphasized in the many studies that show the enhanced expression of CDC25B in a wide variety of cancers (Kristjansdottir et al., 2004). Regulation of the CDC25B phosphatase is therefore of critical importance in controlling cell proliferation. The activity of the CDC25 phosphatases is controlled by factors such as intracellular localization, phosphorylation by CDK1/Cyclin B1 complexes. Activation of p38 signaling pathway results in the activation of MAPKAP kinase-2 in response to stress from UV and cycloheximide (Bulavin et al., 2001; Lindqvist et al., 2004; Manke et al., 2005). MAPKAP kinase-2 binds to and phosphorylates CDC25B and this mediates the subsequent binding of 14-3-3 and the shuttling of CDC25B from nucleus into cytoplasm, which together with 14-3-3 induce G2/M delay (Bulavin et al., 2001; Lindqvist et al., 2004; Manke et al., 2005). A specific inhibitor of p38 has been shown to significantly reduce CDC25B/14-3-3 interactions and initiate the G2/M checkpoint response (Bulavin et al., 2001). Our study demonstrated that the proportion of CDC25B in nucleus and cytoplasm was disrupted in ZS NHBE cells with lower amount of CDC25B protein in nucleus and higher amount of CDC25B protein in cytoplasm than in ZN control cells **(Fig. 5.7)**. This displacement of CDC25B from nucleus to cytoplasm may account for the G2/M arrest in ZS NHBE cells. Thereafter, we investigated the dependency of CDC25 shuttling on p38 MAPK activity by suppressing the activation of p38 MAPK by

dominant negative and inhibitor SB202190. The present finding indicated that after suppression of p38 MAPK, the markedly elevated amount of cytoplasmic CDC25B in ZS cells was restored back to normal level as compared to ZN control cells, providing supportive evidence to show that the distorted shuttling of CDC25B was due to the activation of p38 MAPK which lead to and caused the delay in G2/M in ZS NHBE cells.

Taken together, we report for the first time that the increase of p38 kinase activity and mRNA expression induced the delay in G2/M in ZS NHBE cells. In addition, the observed upregulation of Gadd45, accumulation of phosphorylated p53 at serine 15, reduction of the CDK1 and Cyclin B1 complex formation and shuttling of CDC25B to the cytoplasm are downstream events of the p38 MAPK signal pathway which results in the delay in G2/M progression.

CHAPTER VI:

SUMMARY

The project was designed to examine the effect of cellular zinc status on the signal transduction of tumor suppressor genes. Data generated support the induction of a signal transduction pathway involving Gadd45, p53 and p38 MAPK in the delay of G2/M progression in zinc supplemented (ZS) cells **(Fig.6.1).** This figure presents a possible overall signal pathway to summarize the cellular events constructed from the present findings. In ZS NHBE cells, the level of reactive oxygen species (ROS) was found to be approximately 20% higher than that of ZN control cells (Shih et al., 2007). The ROS generated by stress has been reported to induce the membrane receptors to trigger a series of cell signal reactions to protect cells from damage (Moiseeva et al., 2006). In our study, the ROS generated in high zinc stress may initiate the downstream cell signal transduction and induce the activation of protein kinases, such as MTK1, resulting in the activation of p38 MAPK. Environmental stresses, including UV radiation, ionizing radiation and osmotic stress have been shown to activate the p38 MAPK pathway (Takekawa et al., 1998). In the ZS cells, the enhanced activation of p38 MAPK upregulated Gadd45 expression, displaced CDC25B from nucleus to cytoplasm, reduced cellular levels of CDK1/Cyclin B1 complex, enhanced the activation of phosphorylation of p53 at serine 15, and delayed G2/M cell cycle progression, were returned back to similar level of ZN control cells by knocking down or suppressing p38 with dominant negative or protein inhibitor. Thus, these zinc supplementation induced cellular events appeared to be p38 MAPK dependent.

Regulation of Gadd45 induction after DNA damage is complex and may be involved

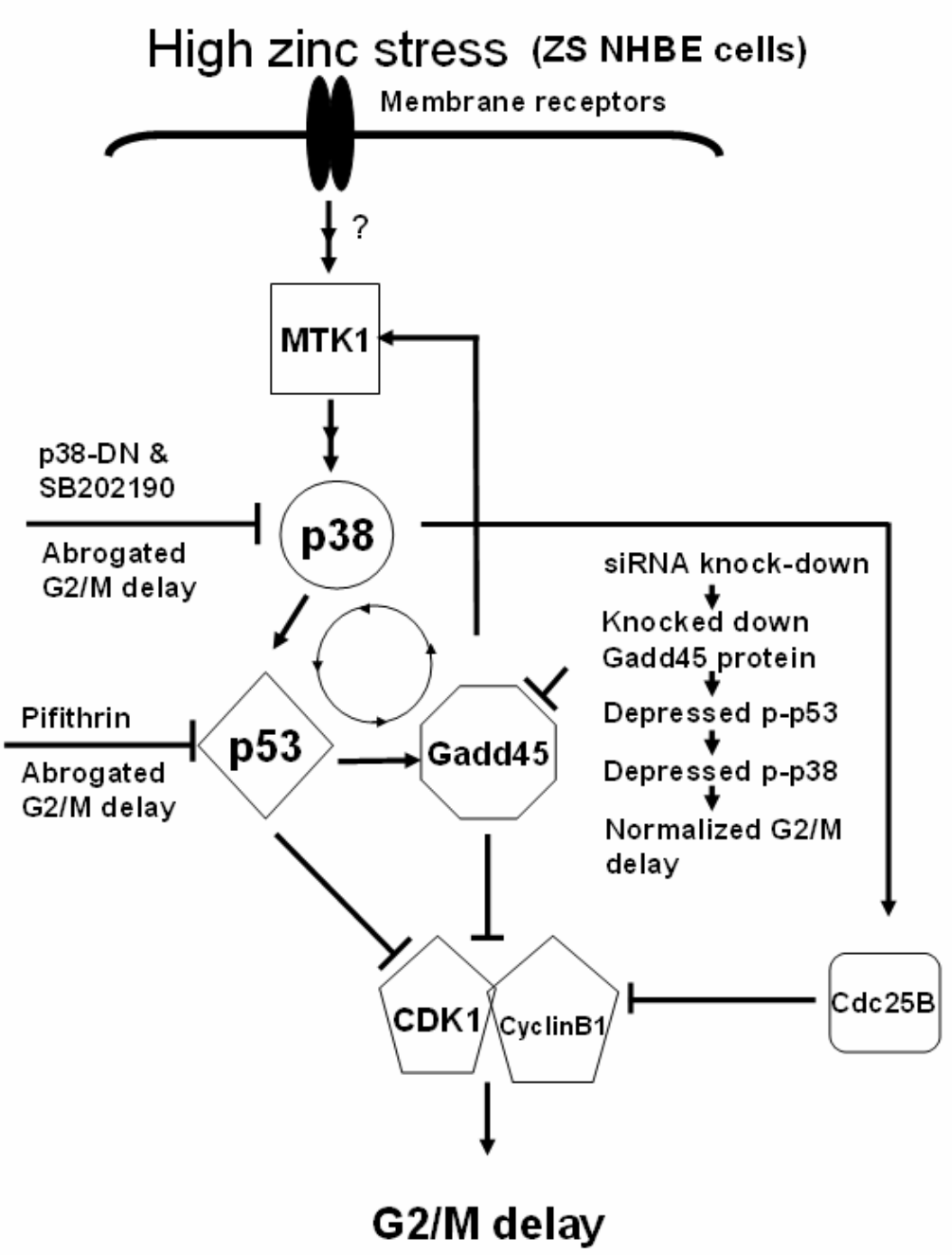
in p53-dependent and –independent signaling pathways. Gadd45 induction by IR has been reported to be strictly dependent on normal cellular p53 function. p53 has been established to enhance Gadd45 expression and reduce the formation of CDK1/Cyclin B1 complex and eventually arrest cells in G2/M progression in response to stress (Zhan et al., 1999). Similarly, the zinc supplementation induced inhibition of cell growth, up-regulation of p53 mRNA and protein expressions, and blockage of G2/M cell cycle progression were normalized by the administration of p53 protein inhibitor, Pifithrin, which inhibited p53 transactivation activity. After the inhibition of p53 transactivation activity, the enhanced Gadd45 promoter activity, upregulated protein expression, reduction in the cellular level of CDK1/Cyclin B1 complex, and delay in G2/M cell cycle progression were normalized in ZS NHBE cells, implicating that the associated changes in this regulatory pathway are p53 dependent.

Furthermore, the enhanced Gadd45 mRNA and protein expression, and enhanced phosphorylated p53 (ser15) and phosphorylated p38, blockage in G2/M cell cycle were partially normalized after the knock down of Gadd45 mediated by the RNA interference (siRNA) approach, which demonstrated that the G2/M blockage is only partially dependent on Gadd45. Our results are consisted with previous finding that showed Gadd45 was implicated in G2/M arrest in response to UV, IR or MMS stresses (Fornace et al., 1989; Zhan et al., 1999). Furthermore, our findings support the involvement of a positive feedback loop consisting of Gadd45, p53 and p38 MAPK, in response to stress induced by zinc supplementation. Similarly, other studies have shown that upregulation of Gadd45 is capable of enhancing p53 protein level through a positive feedback loop by binding to MTK1 and activating p38 indirectly (Jin et al., 2003; Takekawa et al, 1998).

Taken together, we reported for first time that a reduction in cell growth, delay in G2/M progression, decrease in the level of CDK1/Cyclin B1 complex, displacement of CDC25B from nucleus to cytoplasm, enhancement of Gadd45, p53, p38 MAPK mRNA and protein expressions, as well as increases in phosphorylation of p53 and p38 MAPK, were Gadd45, p53 and p38 MAPK dependent in zinc supplemented NHBE cells. The cytotoxicity induced by high zinc stress may trigger the signal cascade composed of p38-p53-Gadd45-CDK1/Cyclin B1 and delay G2/M progression.

A better understanding of the mechanisms involved in the protection of normal cells from zinc cytotoxicity is essential for the identification of potential targets, such as Gadd45, p53 and p38, and may contribute to the development of future therapeutic approaches for the treatment of human lung diseases.

Fig.6.1. Possible pathway responsible for the delay in G2/M progression in ZS NHBE cells.



BIBLIOGRAPHY

Adams WJ, Conard B, Ethier G, Brix KV, Paquin PR, and DiToro DM. The challenges of hazard identification and classification of insoluble metals and metal substances for the aquatic environment. Human and Ecological Risk Assessment 6: 1019-1038, 2000.

Agarwal ML, Agarwal A, Taylor WR, and Stark GR. p53 controls both the G2/M and the G/1 cell-cycle checkpoint and mediates reversible growth arrest in human fibroblasts. Proceedings of the National Academy of Sciences of the United States of America 92: 8493-8497, 1995.

Ambrosino C and Nebreda AR. Cell cycle regulation by p38 MAP kinases. Biology of the Cell 93: 47-51, 2001.

Agren MS. Zinc in wound repair. Archives of Dermatology 135: 1273-1274, 1999.

Apodaca G. Endocytic traffic in polarized epithelial cells: Role of the actin and microtubule cytoskeleton. Traffic 2: 149-159, 2001.

Arsenault JE and Brown KH. Zinc intake of US preschool children exceeds new dietary reference intakes. American Journal of Clinical Nutrition 78: 1011-1017, 2003.

Asokananthan N, Graham PT, Stewart DJ, Bakker AJ, Eidne KA, Thompson PJ, and Stewart GA. House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: The cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. Journal of Immunology 169: 4572-4578, 2002.

Ayers MM and Jeffery PK. Proliferation and differentiation in mammalian airway epithelium. European Respiratory Journal 1: 58-80, 1988.

Bataineh ZM, Bani-Hani IH, Al-Alami JR, Zinc in normal and pathological human prostate gland, Saudi Med J. 3: 218–220, 2002.

Blanc P, Wong H, Bernstein MS, and Boushey HA. An experiemtnatl human-model of metal fume fever. Annals of Internal Medicine 114: 930-936, 1991.

Bruinsma JJ, Jirakulaporn T, Muslin AJ, and Kornfeld K. Zinc ions and cation diffusion facilitator proteins regulate Ras-mediated signaling. Developmental Cell 2: 567-578, 2002.

Bucchieri F, Puddicombe SM, Lordan JL, Richter A, Buchanan D, Wilson SJ, Ward J, Zummo G, Howarth PH, Djukanovic R, Holgate ST, and Davies DE. Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. American Journal of Respiratory Cell and Molecular Biology 27: 179-185, 2002.

Bulavin DV, Kovalsky O, Hollander MC, and Fornace AJ. Loss of oncogenic H-ras-induced cell cycle arrest and p38 mitogen-activated protein kinase activation by disruption of gadd45a. Molecular and Cellular Biology 23: 3859-3871, 2003.

Bulavin DV, Demidov ON, Saito S, Kauraniemi P, Phillips C, Amundson SA, Ambrosino C, Sauter G, Nebreda AR, Anderson CW, Kallioniemi A, Fornace AJ, and Appella E. Amplification of PPM1D in human tumors abrogates p53 tumor-suppressor activity. Nature Genetics 31: 210-215, 2002.

Bulavin DV, Saito S, Hollander MC, Sakaguchi K, Anderson CW, Appella E, and Fornace AJ. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. Embo Journal 18: 6845-6854, 1999.

Bulavin DV, Higashimoto Y, Popoff IJ, Gaarde WA, Basrur V, Potapova O, Appella E, Fornace AJ Jr. Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. Nature 411:102-107, 2001.

Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, and Vogelstein B. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 282: 1497-1501, 1998.

Carrier F, Bae I, Smith ML, Ayers DM, and Fornace AJ. Characterization of the GADD45 response to ionizing radiation in WI-L2-NS cells, a p53 mutant cell line. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis 352: 79-86, 1996.

Carrier F, Georgel PT, Pourquier P, Blake M, Kontny HU, Antinore MJ, Gariboldi M, Myers TG, Weinstein JN, Pommier Y, and Fornace AJ. Gadd45, a p53-responsive stress protein, modifies DNA accessibility on damaged chromatin. Molecular and Cellular Biology 19: 1673-1685, 1999.

Carter JE, Truong-Tran AQ, Grosser D, Ho L, Ruffin RE, and Zalewski PD. Involvement of redox events in caspase activation in zinc-depleted airway epithelial cells. Biochemical and Biophysical Research Communications 297: 1062-1070, 2002.

Cataldo DD, Gueders MM, Rocks N, Sounni NE, Evrard B, Bartsch P, Louis R, Noel A, and Foidart JM. Pathogenic role of matrix metalloproteases and their inhibitors in asthma and chronic obstructive pulmonary disease and therapeutic relevance of matrix metalloproteases inhibitors. Cellular and Molecular Biology 49: 875-884, 2003.

Chen BW, Wang HH, Liu JX, and Liu XG. Zinc sulphate solution enema decreases inflammation in experimental colitis in rats. Journal of Gastroenterology and Hepatology 14: 1088-1092, 1999.

Chen CY, Oliner JD, Zhan QM, Fornace AJ, Vogelstein B, and Kastan MB. Interactions between p53 and MDM1 in a mammalian-cell cycle checkpoint pathway. Proceedings of the National Academy of Sciences of the United States of America 91: 2684-2688, 1994.

Chesters JK and Boyne R. Nature of the Zn^{2+} requirement for DNA-synthesis by 3T3 cells. Experimental Cell Research 192: 631-634, 1991.

Cho CH, Dai S, and Ogle CW. Effect of Zinc on anaphylaxis in vivo in guinea-pig. British Journal of Pharmacology 60: 607-608, 1977.

Chou TT, Trojanowski JQ, and Lee VMY. p38 mitogen-activated protein kinase-independent induction of gadd45 expression in nerve growth factor-induced apoptosis in medulloblastomas. Journal of Biological Chemistry 276: 41120-41127, 2001.

Cohn L, Elias JA, and Chupp GL. Asthma: Mechanisms of disease persistence and progression. Annual Review of Immunology 22: 789-815, 2004.

Cole AM, Dewan P, and Ganz T. Innate antimicrobial activity of nasal secretions. Infection and Immunity 67: 3267-3275, 1999.

Cui L, Takagi Y, Sando K, Wasa M, and Okada A. Nitric oxide synthase inhibitor attenuates inflammatory lesions in the skin of zinc-deficient rats. Nutrition 16: 34-41, 2000.

Cui L, Takagi Y, Wasa M, Sando K, Khan J, and Okada A. Nitric oxide synthase inhibitor attenuates intestinal damage induced by zinc deficiency in rats. Journal of Nutrition 129: 792-798, 1999.

Deaton CM, Marlin DJ, Smith NC, Smith KC, Newton RJ, Gower SM, Cade SM, Roberts CA, Harris PA, Schroter RC, and Kelly FJ. Breath condensate hydrogen peroxide correlates with both airway cytology and epithelial lining fluid ascorbic acid concentration in the horse. Free Radical Research 38: 201-208, 2004.

Dorscheid DR, Wojcik KR, Sun S, Marroquin B, and White SR. Apoptosis of airway epithelial cells induced by corticosteroids. American Journal of Respiratory and Critical Care Medicine 164: 1939-1947, 2001.

Dreosti IE. Recommended dietary intakes of iron, zinc, and other inorganic nutrients and their chemical form and bioavailability. Nutrition 9: 542-545, 1993.

Eldeiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, and Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. Cell 75: 817-825, 1993.

Elmes ME and Jones JG. Ultrastrutural-changes in the small intestinal of zinc-deficient rats. Journal of Pathology 130: 37-&, 1980.

Estevez AG, Crow JP, Sampson JB, Reiter C, Zhuang YX, Richardson GJ, Tarpey MM, Barbeito L, and Beckman JS. Induction of nitric oxide-dependent apoptosis in motor neurons by zinc-deficient superoxide dismutase. Science 286: 2498-2500, 1999.

Fan WH, Jin SQ, Tong T, Zhao HC, Fan FY, Antinore MJ, Rajasekaran B, Wu M, and Zhan QM. BRCA1 regulates GADD45 through its interactions with the OCT-1 and CAAT motifs. Journal of Biological Chemistry 277: 8061-8067, 2002.

Fanzo JC, Reaves SK, Cui LB, Zhu L, Wu JYJ, Wang YR, and Lei KY. Zinc status affects p53, gadd45, and c-fos expression and caspase-3 activity in human bronchial epithelial cells. American Journal of Physiology-Cell Physiology 281: C751-C757, 2001.

Feng P, Liang JY, Li TL, Guan ZX, Zou J, Franklin R, Costello LC, Zinc induces mitochondria apoptogenesis in prostate cells. Mol Urol. 4: 31–36, 2000.

Fernande.F, Prasad AS, and Oberleas D. Effect of zinc deficiency on nucleic acids, collagen, and noncollagenous protein of connective tissue. Journal of Laboratory and Clinical Medicine 82: 951-961, 1973.

Fletcher C and Peto R. Natural history of chronic air flow obstruction. British Medical Journal 1: 1645-1648, 1977.

Folkerts G and Nijkamp FP. Airway epithelium: more than just a barrier! Trends in Pharmacological Sciences 19: 334-341, 1998.

Fornace AJ, Alamo I, and Hollander MC. DNA damage inducible transcripts in mammalian-cells. Proceedings of the National Academy of Sciences of the United States of America 85: 8800-8804, 1988.

Fornace AJ, Jackman J, Hollander MC, Hoffmanliebermann B, and Liebermann DA. Genotoxic stress response genes and growth arrest genes Gadd, Myd, and other genes induced by treatments eliciting growth arrest. Annals of the New York Academy of Sciences 663: 139-153, 1992.

Fornace AJ, Nebert DW, Hollander MC, Luethy JD, Papathanasiou M, Fargnoli J, and Holbrook NJ. Mammalian genes coordinately regulated by growth arrest signals and DNA damage agents. Molecular and Cellular Biology 9: 4196-4203, 1989.

Fosmire GJ. Zinc toxicity. American Journal of Clinical Nutrition 51: 225-227, 1990.

Frederickson CJ. Neurology of zinc and zinc containing neurons. International Review of Neurobiology 31: 145-238, 1989.

Fuortes L and Schenck D. Marked elevation of urinary zinc levels and pleural-friction rub in metal fume fever. Veterinary and Human Toxicology 42: 164-165, 2000.

Gonul B, Soylemezoglu T, Babul A, and Celebi N. Effects of epidermal growth factor dosage forms on mice full-thickness skin wound zinc levels and relation to wound strength. Journal of Pharmacy and Pharmacology 50: 641-644, 1998.

Hartwell LH and Weinert TA. Checkpoints controls that ensure the order of cell cycle events. Science 246: 629-634, 1989.

Hambidge KM. Hair analyses. Pediatric Clinics of North America 27: 855-860, 1980.

Hambidge M. Human zinc deficiency. Journal of Nutrition 130: 1344S-1349S, 2000.

Harper SJ and LoGrasso P. Signalling for survival and death in neurones - The role of stress-activated kinases, JNK and p38. Cellular Signalling 13: 299-310, 2001.

Hennig B, Meerarani P, Ramadass P, Toborek M, Malecki A, Slim R, and McClain CJ. Zinc nutrition and apoptosis of vascular endothelial cells: Implications in atherosclerosis. Nutrition 15: 744-748, 1999.

Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW, Vogelstein, 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression, Molecular Cell 1: 3-11, 1997.

Hildesheim J, Belova GI, Tyner SD, Zhou XW, Vardanian L, and Fornace AJ. Gadd45a regulates matrix metalloproteinases by suppressing Delta Np63 alpha and beta-catenin via p38 MAP kinase and APC complex activation. Oncogene 23: 1829-1837, 2004.

Hildesheim J, Bulavin DV, Anver MR, Alvord WG, Hollander MC, Vardanian L, and Fornace AJ. Gadd45a protects against UV irradiation-induced skin tumors, and promotes apoptosis and stress signaling via MAPK and p53. Cancer Research 62: 7305-7315, 2002.

Hirose T, Sowa Y, Takahashi S, Saito S, Yasuda C, Shindo N, Furuichi K, and Sakai T. p53-independent induction of Gadd45 by histone deacetylase inhibitor: coordinate regulation by transcription factors Oct-1 and NF-Y. Oncogene 22: 7762-7773, 2003.

Holgate ST. The epidemic of asthma and allergy. Journal of the Royal Society of Medicine 97: 103-110, 2004.

Holgate ST, Lackie PM, Davies DE, Roche WR and Walls AF. The bronchial epithelium as a key regulator of airway inflammation and remodeling in asthma. Clinic Experimental Allergy 29: 90–95, 1999.

Ho LH, Ruffin RE, Murgia C, Li LX, Krilis SA, and Zalewski PD. Labile zinc and zinc transporter ZnT4 in mast cell granules: Role in regulation NF-KB translocation. Journal of Immunology 172: 7750-7760, 2004.

Hollander MC, Kovalsky O, Salvador JM, Kim KE, Patterson AD, Raines DC, and Fornace AJ. Dimethylbenzanthracene carcinogenesis in Gadd45-null mice is associated with decreased DNA repair and increased mutation frequency. Cancer Research 61: 2487-2491, 2001.

Hollander MC, Sheikh MS, Bulavin DV, Lundgren K, Augeri-Henmueller L, Shehee R, Molinaro TA, Kim KE, Tolosa E, Ashwell JD, Rosenberg MP, Zhan QM, Fernandez-Salguero PM, Morgan WF, Deng CX, and Fornace AJ. Genomic instability in Gadd45a-deficient mice. Nature Genetics 23: 176-184, 1999.

Hsu PC and Guo YLL. Antioxidant nutrients and lead toxicity. Toxicology 180: 33-44, 2002.

Iwata M, Takebayashi T, Ohta H, Alcalde RE, Itano Y, and Matsumura T. Zinc accumulation and metallothionein gene expression in the proliferating epidermis during wound healing in mouse skin. Histochemistry and Cell Biology 112: 283-290, 1999.

Jackson JG and Pereira-Smith OM. p53 is Preferentially Recruited to the Promoters of Growth Arrest Genes p21 and GADD45 during Replicative Senescence of Normal Human Fibroblasts. Cancer Research 66: 8356-8360, 2006.

Jaiswal AS and Narayan S. Zinc stabilizes adenomatous polyposis coli (APC) protein levels and induces cell cycle arrest in colon cancer cells. Journal of Cell Biochemistry 93: 345-57, 2004.

Jiang F, Li P, Fornace AJ Jr, Nicosia SV, and Bai W. G2/M arrest by 1,25-dihydroxyvitamin D3 in ovarian cancer cells mediated through the induction of GADD45 via an exonic enhancer. J Biol Chem. 278: 48030-48040, 2003.

Jin SK and Levine AJ. The p53 functional circuit. Journal of Cell Science 114: 4139-4140, 2001.

Jin SQ, Antinore MJ, Lung FT, Dong X, Zhao HC, Fan FY, Colchagie AB, Blanck P, Roller PP, Fornace AJ, and Zhan QM. The GADD45 inhibition of Cdc2 kinase correlates with GADD45-mediated growth suppression. Journal of Biological Chemistry 275: 16602-16608, 2000.

Jin SQ, Fan FY, Fan WH, Zhao HC, Tong T, Blanck P, Alomo I, Rajasekaran B, and Zhan QM. Transcription factors Oct-1 and NF-YA regulate the p53-independent induction of the GADD45 following DNA damage. Oncogene 20: 2683-2690, 2001.

Jin SQ, Tong T, Fan WH, Fan FY, Antinore MJ, Zhu XC, Mazzacurati L, Li XX, Petrik KL, Rajasekaran B, Wu M, and Zhan QM. GADD45-induced cell cycle G2-M arrest associates with altered subcellular distribution of cyclin B1 and is independent of p38 kinase activity. Oncogene 21: 8696-8704, 2002.

Jin SQ, Zhao HC, Fan FY, Blanck P, Fan WH, Colchagie AB, Fornace AJ, and Zhan QM. BRCA1 activation of the GADD45 promoter. Oncogene 19: 4050-4057, 2000.

Jin SQ, Mazzacurati L, Zhu XC, Tong T, Song YM, Shao SJ, Petrik KL, Rajasekaran B, Wu M, and Zhan Q.M. Gadd45a contributes to p53 stabilization in response to DNA damage. Oncogene 22: 8536-8540, 2003.

Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, and Craig RW. Participation of p53 protein in the cellular response to DNA damage. Cancer Research 51: 6304-6311, 1991.

Kastan MB, Zhan QM, Eldeiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B, and Fornace AJ. A mammalian cell cycle checkpoint pathway utilizing p53 and Gadd45 is defective in ataxia telangiectais. Cell 71: 587-597, 1992.

Kaye P, Young H, and O'Sullivan I. Metal fume fever: a case report and review of the literature. Emergency Medicine Journal 19: 268-269, 2002.

Kazantsev A and Sancar A. Does the p53 up-regulated Gadd45 protein have a role in excision repair. Science 270: 1003-1004, 1995.

Kearsey JM, Coates PJ, Prescott AR, Warbrick E, and Hall PA. Gadd45 is a nuclear cell cycle regulated protein which interacts with p21 (CIP1). Oncogene 11: 1675-1683, 1995.

Kearsey JM, Shivji MKK, Hall PA, and Wood RD. Does the p53 upregulated Gadd45 protein have a role in excision repair. Science 270: 1004-1005, 1995.

Knight DA, Lane CL, and Stick SM. Does aberrant activation of the epithelial-mesenchymal trophic unit play a key role in asthma or is it an unimportant sideshow? Current Opinion in Pharmacology 4: 251-256, 2004.

Krajewska M, Wang HG, Krajewski S, Zapata JM, Shabaik A, Gascoyne R, and Reed JC. Immunohistochemical analysis of in vivo patterns of expression of CPP32 (Caspase-3), a cell death protease. Cancer Research 57: 1605-1613, 1997.

Krebs NF. Dietary zinc and iron sources, physical growth and cognitive development of breastfed infants. Journal of Nutrition 130: 358S-360S, 2000.

Krebs NF, Westcott JE, Arnold TD, Kluger BM, Accurso FJ, Miller LV, and Hambidge KM. Abnormalities in zinc homeostasis in young infants with cystic fibrosis. Pediatric Research 48: 256-261, 2000.

Kristjansdottir K, Rudolph J. Cdc25 phosphatases and cancer, Chemical Biology 11: 1043-1051, 2004.

Kültz D, Madhany S, and Burg MB. Hyperosmolality Causes Growth Arrest of murine kidney cells induction of Gadd45 and Gadd153 by osmosensing via protein kinase 2. J Biol Chem. 273: 13645-13651, 1998.

Lambein F, Haque R, Khan JK, Kebede N, and Kuo YH. From soil to brain zinc –deficiency increases the neurotoxicity of lathyrus sativus and may affect the susceptibility for the motor neurone disease neuronlathyrism. Toxicon 32: 461-466, 1994.

Larsen GL, White CW, Takeda K, Loader JE, Nguyen DDH, Joetham A, Groner Y, and Gelfand EW. Mice that overexpress Cu/Zn superoxide dismutase are resistant to allergen-induced changes in airway control. American Journal of Physiology-Lung Cellular and Molecular Physiology 279: L350-L359, 2000.

Leitzmann MF, Stampfer MJ, Wu K, Colditz GA, Willett WC, and Giovannucci EL. Zinc supplement use and risk of prostate cancer. J Natl Cancer Inst 95: 1004–1007, 2003.

Leonard A, Gerber GB, and Leonard F. Mutagenicity, carcinogenicity and teratogenicity of zinc. Mutation Research 168: 343-353, 1986.

Leung CH, Lam W, Zhuang WJ, Wong NS, and Wang WF. PKC delta-dependent deubiquitination and stabilization of Gadd45 in A431 cells overexposed to EGF. Biochemical and Biophysical Research Communications 285: 283-288, 2001.

Levine AJ. p53, the cellular gatekeeper for growth and division. Cell 88: 323-331, 1997.

Liang JY, Liu YY, Zou J, Franklin RB, Costello LC, and Feng P. Inhibitory effect of zinc on prostatic carcinoma cell growth. Prostate 40: 200-207, 1999.

Lindqvist A, Kallstrom H, Rosenthal KC. Characterisation of Cdc25B localisation and nuclear export during the cell cycle and in response to stress. Journal of Cell Science 117:4979-4990., 2004.

Lonnerdal B. Dietary factors influencing zinc absorption. Journal of Nutrition 130: 1378S-1383S, 2000.

Lu B F, Yu H, Chow CW, Li BY, Zheng WP, Davis RJ, and Flavell RA. GADD45γ Mediates the Activation of the p38 and JNK MAP Kinase Pathways and Cytokine Production in Effector TH1 Cells. Immunity 14: 583-590, 2001.

Maeda T, Hanna AN, Sim AB, Chua PP, Chong MT, and Tron,VA. GADD45 regulates G2/M arrest, DNA repair, and cell death in keratinocytes following ultraviolet exposure. J Invest Dermatol. 119: 22-26, 2002.

Maheswaran S, Park S, Bernard A, Morris JF, Rauscher FJ, Hill DE, and Haber DA. Physical and functional interaction between WT1 and p53 proteins. Proceedings of the National Academy of Sciences of the United States of America 90: 5100-5104, 1993.

Mak SK and Kultz D. Gadd45 proteins induce G2/M arrest and modulate apoptosis in kidney cells exposed to hyperosmotic stress. J Biol Chem. 279: 39075-39084, 2004.

Malo JL, Cartier A, and Dolovich J. Occupational asthma due to zinc. European Respiratory Journal 6: 447-450, 1993.

Manke IA, Nguyen A, Lim D, Stewart MQ, Elia AE, Yaffe MB. MAPKAP kinase-2 is a cell cycle checkpoint kinase that regulates the G2/M transition and S phase progression in response to UV irradiation. Molecular Cell 17:37-48, 2005.

Martins RJE and Boaventura RAR. Uptake and release of zinc by aquatic bryophytes (Fontinalis antipyretica L. ex. Hedw.). Water Research 36: 5005-5012, 2002.

Merchant J and Webby R. Metal fume fever: a case report and literature review. Emerg Med Fremantle. 13: 373– 375, 2001.

Mita H, Tsutsui J, Takekawa M, Witten EA, and Saito H. Regulation of MTK1/MEKK4 kinase activity by its N-terminal autoinhibitory domain and GADD45 binding. Molecular and Cellular Biology 22: 4544-4555, 2002.

Miyake Z, Takekawa M, Ge Q, and Saito H. Activation of MTK1/MEKK4 by GADD45 through induced N-C dissociation and dimerization-mediated trans-autophosphorylation of the MTK1 kinase domain. Mol. Cell. Biol. Jan 22 [Epub ahead of print]. 2007.

Miyashita T and Reed JC. Tumor-suppressor p53 is a direct transcriptional activator of the human Bax gene.Cell 80: 293-299, 1995.

Muyssen BTA and Janssen CR. Accumulation and regulation of zinc in Daphnia magna: Links with homeostasis and toxicity. Archives of Environmental Contamination and Toxicology 43: 492-496, 2002.

Mullan PB, Quinn JE, Gilmore PM, McWilliams S, Andrews H, Gervin C, McCabe N, McKenna S, White P, Song YH, Maheswaran S, Liu E, Haber DA, Johnston PG, and Harkin DP. BRCA1 and GADD45 mediated G2/M cell cycle arrest in response to antimicrotubule agents. Oncogene 20: 6123-6131, 2001.

Morgan DO, Principles of CDK regulation. Nature 374: 131–134, 1995.

Morgan DO. Cyclin-dependent kinases: Engines, clocks, and microprocessors, Annual Review of Cell and Developmental Biology13: 261-291, 1997

Novick SG, Godfrey JC, Pollack RL, and Wilder HR. Zinc-induced suppression of inflammation in the respiratory tract, caused by infection with human rhinovirus and other irritants. Medical Hypotheses 49: 347-357, 1997.

Nyberg KA, Michelson RJ, Putnam CW, and Weinert TA. Toward maintaining the genome: DNA damage and replication checkpoints. Annual Review of Genetics 36: 617-656, 2002.

Ohtani-Fujita N, Minami S, Mimaki S, Dao S, and Sakai T. p53-independent activation of the Gadd45 promoter by Delta12-prostaglandin J2. Biochem. Biophys. Res. Commun. 251: 648–652, 1998.

Oh-Hashi K, Maruyama W, and Isobe K. Peroxynitrite induces GADD34, 45, and 153 VIA p38 MAPK in human neuroblastoma SH-SY5Y cells. Free Radic Biol Med. 30: 213-221, 2001.

O'Prey J, Brown J, Fleming J, and Harrison PR. Effects of dietary flavonoids on major signal transduction pathways in human epithelial cells. Biochem Pharmacol. 66: 2075-2088, 2003.

Oren M. Regulation of the p53 tumor suppressor protein. Journal of Biological Chemistry 274: 36031-36034, 1999.

Paramanantham R, Bay BH, and Sit KH. Flow cytometric evaluation of the DNA profile and cell cycle of zinc supplemented human Chang liver cells. Acta Paediatr Jpn. 38:334-338, 1996.

Papathanasiou MA, Kerr NCK, Robbins JH, McBride OW, Alamo I, Barrett SF, Hickson ID, and Fornace AJ. Induction by ionization radiation of the Gadd45 gene in cultured human cells lack of mediation by protein kinase C. Molecular and Cellular Biology 11: 1009-1016, 1991.

Pories WJ, Henzel JH, Rob CG, and Strain WH. Acceleration of healing with zinc sulfate. Annals of Surgery 165: 432-&, 1967.

Prasad AS. Zinc: an overview. Nutrition 11: 93-99, 1995.

Prasad AS. Zinc: The biology and therapeutics of an ion. Annals of Internal Medicine 125: 142-144, 1996.

Prives C and Hall PA. The P53 pathway. Journal of Pathology 187: 112-126, 1999.

Poon RY, Jiang W, Toyoshima H, Hunter T, Cyclin-dependent kinases are inactivated by a combination of p21 and Thr-14/Tyr-15 phosphorylation after UV-induced DNA damage. Journal of Biological Chemistry 271: 13283–13291, 1996

Richter M, Cantin AM, Beaulieu C, Cloutier A, and Larivee P. Zinc chelators inhibit eotaxin, RANTES, and MCP-1 production in stimulated human airway epithelium and fibroblasts. American Journal of Physiology-Lung Cellular and Molecular Physiology 285: L719-L729, 2003.

Rincon M, Flavell RA, and Davis RA. The JNK and P38 map kinase signaling pathways in T cell-mediated immune responses. Free Radical Biology and Medicine 28: 1328-1337, 2000.

Rogers DF. Airway mucus hypersecretion in asthma: an undervalued pathology? Current Opinion in Pharmacology 4: 241-250, 2004.

Roychowdhury M, Sarkar N, Manna T, Bhattacharyya S, Sarkar T, BasuSarkar P, Roy S, and Bhattacharyya B. Sulfhydryls of tubulin - A probe to detect conformational changes of tubulin. European Journal of Biochemistry 267: 3469-3476, 2000.

Sanchez-Prieto R, Rojas JM, Taya Y, and Gutkind JS. A role for the p38 mitogen-activated protein kinase pathway in the transcriptional activation of p53 on genotoxic stress by chemotherapeutic agents. Cancer Research 60: 2464-2472, 2000.

Saelens X, Festjens N, Vande Walle L, van Gurp M, van Loo G, and Vandenabeele P. Toxic proteins released from mitochondria in cell death. Oncogene 23: 2861-2874, 2004.

Salgueiro MJ, Zubillaga MB, Lysionek AE, Caro RA, Weill R, and Boccio JR. The role of zinc in the growth and development of children. Nutrition 18: 510-519, 2002.

Salvesen GS and Abrams JM. Caspase activation - stepping on the gas or releasing the brakes? Lessons from humans and flies. Oncogene 23: 2774-2784, 2004.

Serrano L, Dominguez JE, and Avila J. Identification of zinc binding sites of proteins: zinc binds to the amino terminal region of tubulin. Analytical Biochemistry 172: 210-218, 1988.

Shaulian E and Karin M. Stress-induced JNK: Activation is independent of Gadd45 induction. Journal of Biological Chemistry 274: 29595-29598, 1999.

Sheikh MS, Hollander MC, and Fornace AJ. Role of Gadd45 in apoptosis. Biochemical Pharmacology 59: 43-45, 2000.

She QB, Bode AM, Ma WY, Chen NY, and Dong ZG. Resveratrol-induced activation of p53 and apoptosis is mediated by extracellular-signal-regulated protein kinases and p38 kinase. Cancer Research 61: 1604-1610, 2001.

She QB, Chen NY, and Dong ZG. ERKs and p38 kinase phosphorylate p53 protein at serine 15 in response to UV radiation. Journal of Biological Chemistry 275: 20444-20449, 2000.

Stewart N, Hicks GG, Paraskevas F, and Mowat M. Evidence for a 2nd cell-cycle block at G2/M by p53. Oncogene 10: 109-115, 1995.

Sisson JH, Papi A, Beckmann JD, Leise KL, Wisecarver J, Brodersen BW, Kelling CL, Spurzem JR, and Rennard SI. Smole and viral infection cause cilia loss detectable by bronchoalbeolar lavage cytology and dynein elisa. American Journal of Respiratory and Critical Care Medicine 149: 205-213, 1994.

Smith ML, Kontny HU, Zhan QM, Sreenath A, Oconnor PM, and Fornace AJ. Antisense GADD45 expression results in decreased DNA repair and sensitizes cells to uv-irradiation or cisplatin. Oncogene 13: 2255-2263, 1996.

Smith ML, Chen IT, Zhan Q, Bae I, Chen CY, Gilmer TM, Kastan MB, O'Connor PM, and Fornace AJ Jr. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science 266: 1376–1380, 1994.

Smith ML and Seo YR. p53 regulation of DNA excision repair pathways. Mutagenesis 17: 149-156, 2002.

Strausfeld U, Labbe JC, Fesquet D, Cavadore JC, Picard A, Sadhu K, Russell P, Doree M. Dephosphorylation and activation of a p34cdc2/cyclin B complex in vitro by human CDC25 protein, Nature 351: 242-245, 1991.

Stumbles PA. Regulation of T helper cell differentiation by respiratory tract dendritic cells. Immunology and Cell Biology 77: 428-433, 1999.

Sutter A P, Maaser K, Barthel B, and Scherubl H. Ligands of the peripheral benzodiazepine receptor induce apoptosis and cell cycle arrest in oesophageal cancer cells: involvement of the p38MAPK signalling pathway. Br J Cancer 89: 564-572, 2003.

Swaminath G, Lee TW, and Kobilka B. Identification of an allosteric binding site for ZN(2+) on the beta(2) adrenergic receptor. Journal of Biological Chemistry 278: 352-356, 2003.

Takahashi S, Saito S, Ohtani N, and Sakai T. Involvement of the Oct-1 regulatory element of the gadd45 promoter in the p53-independent response to ultraviolet irradiation. Cancer Research 61: 1187-1195, 2001.

Takekawa M and Saito H. A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. Cell 95: 521-530, 1998.

Tang HY, Zhao K, Pizzolato JF, Fonarev M, Langer JC, and Manfredi JJ. Constitutive expression of the cyclin-dependent kinase inhibitor p21 is regulated by the tumor suppressor protein p53. J Biol Chem. 273: 29156 – 29163, 1998.

Taylor KM, Morgan HE, Johnson A, Hadley LJ, and Nicholson RI. Structure-function analysis of LIV-1, the breast cancer-associated protein that belongs to a new subfamily of zinc transporters. Biochemical Journal 375: 51-59, 2003.

Thompson PJ. Unique role of allergens and the epithelium in asthma. Clinical and Experimental Allergy 28: 110-116, 1998.

Tong T, Fan WH, Zhao HC, Jin SQ, Fan FY, Blanck P, Alomo I, Rajasekaran B, Liu YS, Holbrook NJ, and Zhan QM. Involvement of the MAP kinase pathways in induction of GADD45 following UV radiation. Experimental Cell Research 269: 64-72, 2001.

Tran H, Brunet A, Grenier JM, Datta SR, Fornace AJ, DiStefano PS, Chiang LW, and Greenberg ME. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science 296: 530-534, 2002.

Truong-Tran AQ, Carter J, Ruffin RE, and Zalewski PD. The role of zinc in caspase activation and apoptotic cell death. Biometals 14: 315-330, 2001.

Truong-Tran AQ, Carter J, Ruffin R, and Zalewski PD. New insights into the role of zinc in the respiratory epithelium. Immunology and Cell Biology 79: 170-177, 2001.

Truong-Tran AQ, Ho LH, Chai F, and Zalewski PD. Cellular zinc fluxes and the regulation of apoptosis/gene-directed cell death. Journal of Nutrition 130: 1459S-1466S, 2000.

Truong-Tran AQ, Ruffin RE, Foster PS, Koskinen AM, Coyle P, Philcox JC, Rofe AM, and Zalewski PD. Altered zinc homeostasis and caspase-3 activity in murine allergic airway inflammation. American Journal of Respiratory Cell and Molecular Biology 27: 286-296, 2002.

Uzzo RG, Leavis P, Hatch W, Gabai VL, Dulin N, Zvartau N, and Kolenko VM. Zinc inhibits nuclear factor-kappaB activation and sensitizes prostate cancer cells to cytotoxic agents. Clin Cancer Res. 8: 3579– 3583, 2002.

Vallee BL and Falchuk KH. The biochemical basis of zinc physiology. Physiological Reviews 73: 79-118, 1993.

Van Eerdewegh P, Little RD, Dupuis J, Del Mastro RG, Falls K, Simon J, Torrey D, Pandit S, McKenny J, Braunschweiger K, Walsh A, Liu ZY, Hayward B, Folz C, Manning SP, Bawa A, Saracino L, Thackston M, Benchekroun Y, Capparell N, Wang M, Adair R, Feng Y, Dubois J, FitzGerald MG, Huang H, Gibson R, Allen KM, Pedan A, Danzig MR, Umland SP, Egan RW, Cuss FM, Rorke S, Clough JB, Holloway JW, Holgate ST, and Keith TP. Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. Nature 418: 426-430, 2002.

Vairapandi M, Balliet AG, Fornace AJ Jr, Hoffman B, and Liebermann DA. The differentiation primary response gene MyD118, related to GADD45, encodes for a nuclear protein which interacts with PCNA and p21WAF1/CIP1. Oncogene 12: 2579–2594, 1996.

Vogelstein B and Kinzler KW. p53 funciton and dysfunction. Cell 70: 523-526, 1992.

Vogelstein B, Lane D, and Levine AJ. Surfing the p53 network. Nature 408: 307-310, 2000.

Waldman T, Kinzler KW, and Vogelstein B. p21 is necessary for the p53 mediated G1 arrest in human cancer cells. Cancer Research 55: 5187-5190, 1995.

Walsh CT, Sandstead HH, Prasad AS, Newberne PM, and Fraker PJ. Zinc helath effects and research priorities for the 1990s. Environmental Health Perspectives 102: 5-46, 1994.

Wang XT, Gorospe M, and Holbrook NJ. gadd45 is not required for activation of c-Jun N-terminal kinase or p38 during acute stress. Journal of Biological Chemistry 274: 29599-29602, 1999.

Wang XW, Zhan QM, Coursen JD, Khan MA, Kontny HU, Yu LJ, Hollander MC, O'Connor PM, Fornace AJ, and Harris CC. GADD45 induction of a G(2)/M cell cycle checkpoint. Proceedings of the National Academy of Sciences of the United States of America 96: 3706-3711, 1999.

White E. Life, death, and the pursuit of apoptosis. Genes & Development 10: 1-15, 1996.

Williams RJP. Zinc: what is its role in biology. Endeavour 8: 65-70, 1984.

Williams DL, Newman TC, Shelness GS, and Gordon DA. Measurement of apolipoprotein mRNA by DNA-excess solution hybridization with single-stranded probes. Methods Enzymol. 128: 671-689, 1986.

Woolcock AJ and Barnes PJ. Asthma-the important questions. 2. From the conference of asthma-the important of questions malta October 4-5, 1991 - American Review of Respiratory Disease 146: 1351-&, 1992.

Wright DT, Cohn LA, Li HF, Fischer B, Li CM, and Adler KB. Interactions of oxygen radicals with airway epithelium. Environmental Health Perspectives 102: 85-90, 1994.

Wu WD, Wang XC, Zhang WL, Reed W, Samet JM, Whang YE, and Ghio AJ. Zinc-induced PTEN protein degradation through the proteasome pathway in human airway epithelial cells. Journal of Biological Chemistry 278: 28258-28263, 2003.

Zalewski PD, Millard SH, Forbes IJ, Kapaniris O, Slavotinek A, Betts WH, Ward AD, Lincoln SF, and Mahadevan I. Video image analysis of labile zinc in viable pancreatic islet cells using a specific fluorescent probe for zinc. Journal of Histochemistry & Cytochemistry 42: 877-884, 1994.

Zauberman A, Barak Y, Ragimov N, Levy N, and Oren M. Sequence specific DNA binding by p53 identification of target sites and lack of binding to p53 MDM2 complexes. EMBO Journal 12: 2799-2808, 1993.

Zhan QM, Antinore MJ, Wang XW, Carrier F, Smith ML, Harris CC, and Forance AJ. Association with Cdc2 and inhibition of Cdc2/cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene 18: 2892-2900, 1999.

Zhan QM, Bae I, Kastan MB, and Fornace AJ. The p53 dependent gamma ray response of Gadd45. Cancer Research 54: 2755-2760, 1994.

Zhan KA, Lord I, Alamo Jr, Hollander MC, Carrier F, Ron D, Kohn KW, Hoffman B, Liebermann DA, and Fornace AJ Jr. The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth, Mol Cell Biol. 14: 2361–2371, 1994.

Zhan QM, Chen IT, Antinore MJ, and Fornace AJ. Tumor suppressor p53 can participate in transcriptional induction of the GADD45 promoter in the absence of direct DNA binding (vol 18, pg 2768, 1998). Molecular and Cellular Biology 18: 5620-5620, 1998.

Zhan QM, Fan SJ, Bae I, Guillouf C, Liebermann DA, Oconnor PM, and Fornace AJ. Induction of Bax by genotoxic stress in human cells correlates with normal p53 status and apoptosis. Oncogene 9: 3743-3751, 1994.

Zhan QM, Fan SJ, Smith ML, Bae I, Yu K, Alamo I, Oconnor PM, and Fornace AJ. Abrogation of p53 function affects gadd gene responses to DNA base-damaging agents and starvation. DNA and Cell Biology 15: 805-815, 1996.

Zhan QM, Jin SQ, Ng B, Plisket J, Shangary S, Rathi A, Brown KD, and Baskaran R. Caspase-3 mediated cleavage of BRCA1 during UV-induced apoptosis. Oncogene 21: 5335-5345, 2002.

Zhao M, New L, Kravchenko VV, Kato Y, Gram H, di Padova F, Olson EN, Ulevitch RJ, and Han JH. Regulation of the MEF2 family of transcription factors by p38. Molecular and Cellular Biology 19: 21-30, 1999.

Zhao HC, Jin SQ, Antinore MJ, Lung FDT, Fan FY, Blanck P, Roller P, Fornace AJ, and Zhan QM. The central region of Gadd45 is required for its interaction with p21/WAF1. Experimental Cell Research 258: 92-100, 2000.

Zhao HC, Jin SQ, Fan FY, Fan WH, Tong T, and Zhan QM. Activation of the transcription factor Oct-1 in response to DNA damage. Cancer Research 60: 6276-6280, 2000.

Zsembery A, Fortenberry JA, Liang LH, Bebok Z, Tucker TA, Boyce AT, Braunstein GM, Welty E, Bell PD, Sorscher EJ, Clancy JP, and Schwiebert EM. Extracellular zinc and ATP restore chloride secretion across cystic fibrosis airway epithelia by triggering calcium entry. Journal of Biological Chemistry 279: 10720-10729, 2004.