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Normal human skin fibroblast cells were used to study the effect of zinc supplementation of the media on cell growth and the competitive binding activity of low density lipoprotein (LDL). Cells were grown in the media containing Dulbecco's Modified Eagle Medium (DMEM), 5% (v/v) fetal calf serum (FCS), and various levels of zinc. Cell counts and protein determination revealed that there was no stimulatory effect of zinc on the growth of cells, showing a flat growth curve with up to 6 μg/ml zinc supplementation. However, zinc supplementation of greater than 6 μg/ml to the medium appeared to be toxic to the cell and thereby prevented growth. When zinc was removed from the medium using Epoxy-activated Sepharose 6B coupled with iminodiacetate, zinc concentration in the medium was markedly reduced to 0.045 μg/ml from 0.210 μg/ml. The cell growth study using this zinc depleted medium exhibited a growth curve similar to that obtained from the earlier study, suggesting that 0.045 μg/ml of zinc in the control medium was still sufficient to support normal cell growth. For the LDL binding study, cells were grown in the media with various levels of zinc supplementation for 7 days and the competitive binding activity of LDL was determined. When cells were grown in the zinc removed medium with 1.5 μg/ml zinc supplementation, the maximum amounts of ^{125}I-LDL bound and internalized in the cells were observed; however, higher levels of zinc supplementation to the growth medium

caused decreased ^{125}I-LDL binding to the cell receptors. These results suggest that zinc may be involved in the binding of LDL to the receptors.

Effect of Zinc Supplementation on Cell Growth and Lipoprotein Binding in Human Fibroblast Cells

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INTRODUCTION

A positive correlation between the development of atherosclerosis and increased plasma cholesterol levels has become evident by numbers of epidemiological studies. Low density lipoprotein (LDL) is the major cholesterol carrying lipoprotein in the human plasma. A high level of LDL-cholesterol in the plasma can potentiate the development of atherosclerosis (Brown and Goldstein, 1976). Since discovery of LDL receptors in human fibroblast cells in 1973 (Brown et al., 1973; Goldstein and Brown, 1973), the metabolism of cholesterol has been extensively studied using cell culture. Healthy humans possess an efficient mechanism for the removal of LDL-cholesterol from plasma. This process is mediated by receptors located on the surface of cells in the liver and extrahepatic cells (Brown et al., 1981). Brown and Goldstein (1979) demonstrated the requirement for certain divalent cations, calcium and manganese, for the binding of LDL to the receptors on human fibroblasts. Zinc is an essential trace metal for humans and is functionally and structually important for many enzymes (Vallee, 1976). Zinc is now known to participate in a wide variety of processes including protein, nucleic acid, and carbohydrate metabolism. Although there is very limited information regarding zinc and lipids, it can be hypothesized that zinc may be involved in the binding of LDL to the LDL receptors on human fibroblast cells. This is because metal properties of zinc are similar to those of calcium and manganese, which have been shown to influence LDL binding.

In the present study, the levels of zinc in the medium which influence the normal physiology of human fibroblast cells were first determined. The experiment was designed to study the effect of zinc supplementation of the medium on the growth of cells, expecting to identify deficient, optimal, or toxic effects of zinc. Furthermore, to examine if the zinc status of cells influences receptor mediated

LDL binding to the cells, competition of ^{125}I-LDL with unlabeled LDL for the receptor was investigated in the cells grown in various zinc concentrations. Competition between LDL and other lipoprotein during receptor mediated endocytosis of LDL has been identified (Goldstein and Brown, 1974).

The results of the experiment may provide evidence of zinc involvement in cholesterol metabolism and lead to further studies to reveal the details of the mechanism.

REVIEW OF LITERATURE

Lipoproteins

Lipids circulate in the blood mostly in the form of lipoprotein. Lipoproteins are composed of lipids and specific proteins, called apolipoproteins (Elsenberg and Levy, 1975). Lipoproteins are spherical particles in which an outer polar layer contains phospholipid, apolipoproteins, and free cholesterol. Triglyceride and esterified cholesterol are distributed in the core of the particles (Bradley and Gotto, 1978). The classification of lipoproteins is based on the method of isolation, such as ultracentrifugal separation or electrophoresis (Alfin-Slater and Aftergood, 1980).

Based on the rate of flotation in salt solution (ultracentrifugation), human plasma lipoproteins are generally divided into four classes: chylomicron, very low density lipoprotein (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) (Elsenberg and Levy, 1975). All lipoproteins contain varying amounts of lipids and proteins. The contribution of lipids to the total mass of lipoproteins is more than 98% in the largest chylomicrons and less than 50% in the smallest HDL (Elsenberg and Levy, 1975). These variations affect their density, with chylomicrons being the least dense.

Chylomicrons and VLDL are structually similar (Bradley et al., 1978). In both lipoproteins, more than 95% of the lipid mass are triglycerides (Elsenberg and Levy, 1975). The largest lipoproteins are chylomicrons (diameter > 1000 Å) and have the lowest density (d. < 0.95 g/ml). The next largest lipoproteins are VLDL (diameter is approximately 800 Å) whose density is between 0.95 and 1.006 g/ml. Human plasma LDL have a density interval of 1.006 to 1.063 g/ml. The diameter of the LDL is approximately 220 Å. A nonpolar inner core represents about 50% of the LDL mass and contains cholesterol, predominantly in the esterified form. The rest of the LDL mass is composed of protein and phospholipid, which construct a polar outer

shell (Elsenberg and Levy, 1975). The LDL fraction can be divided into two density fractions: d. 1.006-1.019 g/ml and d. 1.019-1.063 g/ml. The first fraction is called intermediate density lipoprotein (IDL). The density range of HDL is 1.063 to 1.21 g/ml. Approximately 50% of the HDL mass is protein, 30% is phospholipid, and 20% is cholesterol. About 75% of plasma cholesterol is esterified with fatty acids. HDL is usually divided into two density classes: HDL_2 (d. 1.063-1.125 g/ml) and HDL_3 (d. 1.125-1.210 g/ml). HDL_2 has a mean molecular weight of 360,000 and is composed of 60% lipid and 40% protein. The mean molecular weight of HDL_3 is 175,000 and is composed of 45% lipid and 55% apoprotein (Elsenberg and Levy, 1975).

Plasma lipoproteins contain various levels of apoproteins. The more dense the lipoprotein, the more protein content is in the mass (Elsenberg and Levy, 1975). Paper electrophoresis separates lipoproteins: no mobility in chylomicron, pre β-mobility in VLDL, β-mobility in LDL, and α_1-mobility in HDL. Each lipoprotein fraction contains several different apoproteins. According to the ABC nomenclature (Alaupovic, 1971), apoproteins A-I and A-II are the major apoproteins of HDL. Apo A-I activates lecithin-cholesterol acyltransferase (LCAT), whereas apo A-II inhibits this enzyme activity. Apoprotein B is the major protein of LDL and is also present in chylomicrons and VLDL. The lysine (Brown et al., 1981) and arginine (Mahley et al., 1977) residues of apo B are responsible for the binding of LDL to the LDL receptor of the liver and the extrahepatic cells, as well as for the binding of VLDL and HDL to a limited extent. Apo C-II is known to activate extrahepatic lipoprotein lipase, whereas apo C-III inhibits this enzyme activity (Elsenberg and Levy, 1975). Apoprotein E (arginine rich protein) is isolated from VLDL, LDL, and HDL_c. HDL_c is found in the plasma of animals fed high cholesterol diets (Mahley et al., 1976).

Lipoprotein Transport System

Cholesterol in human plasma is derived from two different sources: dietary sources (exogenous) and cellular biosynthesis endogenous) (Brown et al., 1981). Healthy humans possess efficient mechanisms for the removal of cholesterol from plasma through the lipoprotein receptor systems of the hepatic and extrahepatic tissues (Brown et al., 1981). Brown et al. (1981) proposed a model for a lipoprotein transport system for the regulation of plasma cholesterol in humans, illustrating the division between the exogenous and endogenous cycles.

Exogenous Lipid Transport: Most dietary fat is in the form of triglycerides, phospholipids and cholesterol. The total fat intake for many Americans is approximately 150 gm per day, which represents about 40% of the total daily caloric intake (Alfin-Slater and Aftergood, 1980). Typical American adults absorb about 100 grams of triglycerides and 250 milligrams of cholesterol from the diet per day (Brown et al., 1981). In the intestinal tract, these lipids are incorporated into a large lipoprotein, the chylomicron, and secreted into the lymph system. From there chylomicrons enter the blood stream. On the luminal surface of the endothelial cells that line the capillaries of adipose and muscle tissue, triglycerides in the lipoprotein are broken down into fatty acids and glycerols or monoglycerides by lipoprotein lipase. The liberated fatty acids then enter the muscle and adipose tissue for storage or fuel metabolism. Thus, triglycerides are rapidly removed from chylomicron particles, but other lipoprotein components such as cholesteryl ester and apoprotein (apo B, E, and small amount of apo C) remain. As the chylomicrons become smaller particles by the hydrolysis of triglycerides at the peripheral cells, they reenter the blood circulation as chylomicron remnants. The chylomicron remnants are internalized by the liver by lipoprotein receptor mediated endocytosis.

The receptor of the hepatic cells has been known to bind apo B as well as apo E of the chylomicron remnant. Incoming cholesterol esters are converted to free cholesterol in the liver and used for bile acid production, lipoprotein synthesis, or maintenance of the hepatic cell membrane. The bile acids react with nitrogenous bases and yield bile salts (Lehninger, 1978). Most of the bile salts, after being secreted into the small intestine, are reabsorbed from the lower part of the small intestine and returned to the liver, which is known as enterohepatic circulation (Luciano et al., 1978).

Endogenous Lipid Transport: The liver synthesizes triglyceride from carbohydrate and fatty acids, and also synthesizes cholesterol when dietary sources of the sterol are deficient. The liver produces triglyceride rich lipoproteins, VLDL, which are secreted into the blood. At extrahepatic capillary beds, VLDL interacts with lipoprotein lipase and releases most of its triglycerides, thereby becoming a smaller particle. The resulting lipoprotein is called intermediate density lipoprotein (IDL). The excess surface materials, phospholipid and free cholesterol, are taken by HDL in which free cholesterol interacts with lecithin-cholesterol acyl-transferase (LCAT) to form cholesteryl ester. Apoprotein A-I in HDL has been known to activate the LCAT enzyme. The excess free cholesterol is esterified by LCAT with fatty acids derived from the 2-position of lecithin. This newly synthesized cholesteryl ester is transfered back to IDL. The IDL particle, while circulating in the blood, releases triglycerides and all apoproteins except apo B protein. The resulting particles contain almost pure cholesteryl ester in the core and apo B on the surface, and are now called low density lipoprotein (LDL). The LDL transfer their cholesterol to hepatic cells as well as to extrahepatic cells through LDL receptors located on the cell surface.

In the liver, cholesterol esters derived from LDL are used for formation of bile or production of other lipoproteins. In extrahepatic cells, incoming cholesterol esters are hydrolyzed to free cholesterol and reesterified by acyl-CoA cholesterol acyltransferase. The resulting free cholesterol plays a key role in the regulation of cholesterol metabolism in the extrahepatic cells. Details of the mechanism will be discussed later.

When cell turnover occurs, free cholesterol in extrahepatic cells is excreted into the plasma and taken up by HDL and esterified by LCAT. This esterified cholesterol is transfered to IDL and eventually becomes LDL. Then the cycle of LDL metabolism begins again.

Cholesterol and Atherosclerosis

Cholesterol plays important roles in human physiology such as production of bile acids, vitamin D, and steroid hormones, as well as its structural role as a component of the plasma membrane (Alfin-Slater and Aftergood, 1980). However, excessive amounts of cholesterol in the plasma may potentiate the development of atherosclerosis (Brown and Goldstein, 1976).

Atherosclerosis is a disease in the intima of the arteries, especially of the large arteries, in which excessive fat deposit occurs (Guyton, 1981). The development of these lesions is believed to damage the endothelial cells and underlying intima. When the damage has occurred, smooth muscle cells proliferate and migrate from the media of the arteries into the lesions. Shortly after that, excessive amounts of lipids, especially cholesterol, start to deposit in the lesion, forming the atheromatous plaques.

Thus, mammalian cells are faced with the dual problem of providing enough cholesterol for cell membrane and other cellular functions, while at the same time preventing excessive accumulation of cholesterol in the cells (Brown and Goldstein, 1976). It has been proved that this dual task is accomplished in healthy human fibroblast cells (Brown and Goldstein, 1976).

Receptor Mediated Endocytosis of LDL by Human Fibroblasts

The LDL receptor was discovered in 1973 through studies of human skin fibroblasts in tissue culture (Brown et al., 1973; Goldstein and Brown, 1973; and Brown and Goldstein, 1974). Since then, the metabolism of cholesterol has been extensively studied. These studies have led to an enhanced understanding of cholesterol homeostasis on the cellular basis. The most recent advance in biology provides a clear image of the important metabolic event, receptor mediated endocytosis, visualizing it by electron microscopy.

Binding of LDL to the LDL Receptor: Schneider et al. (1980, 1982) have recently purified the LDL receptor from bovine adrenal cortex. The receptor is a glycoprotein with a molecular weight of 164,000. Human fibroblasts can produce a maximum of 20,000 to 50,000 receptors per cell for LDL binding (Brown and Goldstein, 1979). The LDL receptor is probably synthesized on ribosomes attached to the endoplasmic reticulum and glycosylated in the Golgi apparatus. The newly synthesized LDL receptor is inserted into the plasma membrane at a random site, then migrates over the membrane until it reaches a coated pit (Goldstein et al., 1979). Coated pits cover only 2% of the cell surface of fibroblasts; however, they contain 50 to 80% of the LDL receptors (Anderson et al., 1977). The coated regions of plasma membranes are specialized structures that function to carry receptor-bound macromolecules into the cell (Anderson et al., 1977).

Anderson et al. (1982) summarized current studies performed in many laboratories and made a list of the macromolecules which cells internalize by receptor mediated endocytosis. These macromolecules are: transport proteins (such as LDL, chylomicron remnant, yolk proteins,transferrin, transcobalamin II); protein hormones (such as epidermal growth factor, insulin, chorionic gonadotropin, nerve growth factor, β-malanotropin); glycoprotein (such as asialoglyco-proteins, and lysosomal enzymes); other plasma proteins

(such as α_2-macroglobulin and maternal immunoglobulins), certain viruses and toxins.

The recognition site of the LDL receptor for LDL in human fibroblasts is a lysine residue and an arginine residue of apo B protein (Brown and Goldstein, 1979).

Anderson et al. (1977) examined the mechanism of endocytosis of LDL using an electron microscope and reported the internalization process as follows. When human fibroblasts are incubated with LDL containing medium at 4°C, LDL can bind to the LDL receptor but is not internalized by the cell. With increasing time at 37°C, nearly all the receptor-bound LDL are incorporated into the coated endocytic vesicles and these vesicles migrate through the cytoplasm within 1 minute and lose their cytoplasmic coat within 2 minutes. In the next 6 minutes, these vesicles fuse with lysosomes.

Degradation of LDL by the Cells: Within the lysosome the protein component of the LDL is degraded to free amino acids (Goldstein and Brown, 1974). This occurs within 30 minutes after the LDL binding (Brown and Goldstein, 1979). The cholesteryl ester component of LDL is hydrolyzed by a lysosomal acid lipase (Brown and Goldstein, 1976). The resultant unesterified cholesterol is converted into a component of cell membranes (Brown et al., 1975).

The accumulation of unesterified cholesterol leads to three regulatory responses which are essential for the homeostatic control of cholesterol levels within the cells (Brown et al., 1981).

1. It suppresses 3-hydroxy-3-methyglutaryl coenzyme A reductase (HMG-CoA reductase) activity. Cholesterol is formed from acyl-CoA in the cells. The synthesis of mevalonate from 3-hydroxy-3-methylglutaryl CoA, which is catalyzed by HMG-CoA reductase, is the rate-limiting step in the process (Gangl and Ockner, 1975). The end product, cholesterol, has been known to suppress HMG-CoA reductase by inhibiting the synthesis of the enzyme (Stryer, 1975). Thus, accumulation of unesterified cholesterol inside cells turns off cholesterol

synthesis by the cells.

2. Accumulated unesterified cholesterol in the cells activates an acyl-CoA: cholesterol acyltransferase (ACAT). This enzyme esterifies the excess free cholesterol for storage purposes.
3. The synthesis of LDL receptors is suppressed by overaccumulation of cholesterol in the cells. Thus, by lowering the number of receptors, further entry of cholesterol is slowed down.

The importance of the normal function of extrahepatic LDL receptors became significant when it was recognized that the fibroblast cells from familial hyper-cholesterolemic patients (FH patients) have a defect in the gene which codes for the receptors (Brown and Goldstein, 1979). The cells from FH patients do not possess receptor mediated cholesterol uptake; therefore, the clearance of LDL-cholesterol in the plasma is impaired(Brown et al., 1981). Heterozygotes of FH patients have a 2 to 3-fold increase in the plasma LDL-cholesterol level and homozygotes of FH patients have 6 to 10-fold elevation in the plasma LDL-cholesterol level. They usually develop myocardial infarction at an early age (Brown et al., 1981).

The binding of LDL to the LDL receptor in cultured human fibroblasts has been found to express two binding processes: a higher affinity binding process and a lower affinity binding process. The high affinity binding process exhibits saturation kinetics at low concentration of the lipoprotein in the medium. The concentration of LDL at which half-maximal binding occurs in fibroblasts is about 20 μg/ml of LDL protein, which is 1/30 of the concentration of LDL in normal human plasma (Goldstein and Brown, 1975). In addition, competition by related lipoprotein such as VLDL occurs in this binding process. These observations suggest that binding is through specific receptor sites on the cell surface. The low affinity binding process shows no saturation kinetics and no competition by VLDL, thus mediating receptor independent binding. Although normal human fibroblasts possess both processes of binding, fibroblast cells from FH

patients lack the high affinity binding process (Goldstein and Brown, 1974).

Effect of Nutrients on LDL Binding and Degradation

There is very limited information regarding the relationship between the LDL binding system and nutrients other than lipids. Goldstein and Brown (1974) reported dependence on calcium ions for LDL binding in the high affinity binding process (at low concentration of LDL in the medium). The half maximal increase in LDL binding was achieved at 0.5 mM of calcium addition to the medium. A drug, chloroquine, has been known to inhibit intralysosomal hydrolase (Stein et al., 1977). Coetzee et al. (1979) demonstrated that exposure of the chloroquine treated cells to sodium ascorbate stimulated LDL protein degradation. They explained the mode of action as followed; ascorbic acid probably enhances lysosomal hydrolase activity by its reducing properties, which counteracts chloroquine action.

Zinc Biochemistry

For over 100 years, zinc has been known to be essential for the growth of microorganisms (Jennings, 1981). In humans, zinc deficiency was first reported in 1961 in Iranian males (Prasad et al., 1961). Since then, a number of pathological conditions associated with zinc deficiency in humans have been described (Karcioğlu, 1980), and it has become evident that zinc is an essential element for humans (National Research Council, 1980).

The clinical syndrome of zinc deficiency is growth retardation, hypogonadism in males, skin changes, poor appetite, delayed wound healing, and decreased dark adaptation (Jennings, 1981). In healthy humans, serum contains 0.84 to 1.20 μg of zinc per ml(Lyenger et al., 1978). Zinc deficiency is often diagnosed by low serum zinc levels (Prasad, 1979). About 32% of serum is tightly bound to α_2-macroglobulin and 66% is loosely bound to albumin (Subcommittee on Zinc,

1979). The binding site of albumin for zinc has been believed to be the free carboxyl group of a histidine residue (Subcommittee on Zinc, 1979).

Zinc Enzymes: A zinc metalloenzyme is defined as a catalytically active metalloprotein containing stoichiometric amounts of zinc firmly bound to the active site of the enzyme (Vallee, 1955). The zinc atoms in the zinc metalloenzymes are very tightly bound; therefore, they do not dissociate from the protein during purification. When zinc is loosely bound to an enzyme, the zinc lacks chemical and functional specificity in the enzyme. In this case the enzyme is designated as a metal-enzyme complex (Riordan and Vallee, 1976).

The evidence of a specific biological function dependent on the presence of zinc first came in 1940 when Keilin and Mann (1940) found that zinc is an enzymatically essential component of carbonic anhydrase. Fifteen years later the second zinc enzyme, bovine pancreatic carboxypeptidase A, was identified by Vallee and Neurath (1955). Thereafter, a number of zinc metalloenzymes were discovered rapidly and now more than eighty, including related enzymes from different species, have been identified. The majority of the discoveries have been within the past decade (Vallee, 1976). Currently known zinc enzymes are present throughout all phyla and are essential for the function and/or structure of at least one enzyme in each of the 6 major categories; oxidoreductase, transferase, hydrolase, lyase, isomerase and ligase (Li and Vallee, 1980). Some zinc metalloenzymes and proteins are listed in Table 1. Among them, carbonic anhydrase, carboxypeptidases, alkaline phosphatase, alcohol dehydrogenase (Li and Vallee, 1980) and superoxide dismutase (Hsieh and Hsu, 1980) have been isolated from human tissues and erythrocytes.

Table 1. Zinc metalloenzymes

Enzyme	Source
Carbonic anhydrase	Bovine and human erythrocyte[a,b,c]
Carboxypeptidase A	Bovine and human pancreas[a,b,c]
Carboxypeptidase B	Bovine and human pancreas[a,b,c]
Alcohol dehydrogenase	Yeast, Horse and human liver[a,b,c]
Alkaline phosphatase	*E. coli**, Human placenta[a.b.c]
Aldolase	Yeast[a,b]
RNA polymerase	*E. coli*[a,c]
DNA polymerase	*E. coli*[a,c]
Reverse transcriptase	Avian myeloblastosis virus[b,c]
Malate dehydrogenase	Bovine heart[c]
Dipeptidase	Porcine kidney[b,c]
D-lactate cytochrome reductase	Yeast[b,c]
Pyruvate carboxylase	Yeast[b]
Superoxide dismutase	*E. coli*, Yeast, Bovine and human erythrocyte[a]

* *Escherichia coli*

a. Subcommittee on Zinc. (1979). Zinc. pp. 220-221. University Park Press. Maryland.

b. Vallee, B.L. (1976). Zinc biochemistry: a perspective. Trends in Biochemical Science 1: 88-91.

c. Hsu, J. M. (1980). Biochemistry and metabolism of zinc. In "Zinc and Copper in Medicine " (Karcioğlu, Z. A. and R. M. Sarper, eds.), pp. 66-93. Charles C Thomas Publisher, Springfield.

The physiological function of carbonic anhydrase is for the rapid dehydration of bicarbonate in the lungs and the repid hydration of carbon dioxide produced in the body (Hsu, 1980). This enzyme contains a single tightly bound zinc ion which is necessary for the activity of the enzyme (Hsu, 1980). The zinc content of the erythrocytes represents about 85% of the zinc in the blood (Vallee, 1959). Carboxypeptidases are proteolytic enzymes secreted from the pancreas (Hsu, 1980). Removal of the zinc atom from these enzymes inactivates the enzyme (Hsu, 1980). Alkaline phosphatase consists of two identical subunits and contains 4 gm atoms of zinc per mole. This enzyme catalyzes the hydrolysis of a variety of phosphate esters. In mammalian tissues, alkaline phosphatase is usually associated with intracellular lipoprotein membranes (Hsu, 1980). The alcohol dehydrogenases from microorganisms and mammalian liver are a tetramer molecule and contain at least 4 gm atoms of zinc per mole and bind 4 moles of nicotineamide adenine dinucleotide (NAD). Human liver alcohol dehydrogenase oxidizes ethanol, methanol and ethylene-glycol (Hsu, 1980). Superoxide dismutase contains 2 gm atoms each of copper and zinc per mole of protein (Subcommittee on Zinc, 1979). Copper is believed to be necessary for the enzyme to function, while zinc is structural in function (Hsieh and Hsu, 1980). This enzyme catalyzes the breakdown of superoxide radicals to oxygen and hydrogen peroxide, thereby protecting living cells against the toxic effect of oxygen (Li and Vallee, 1980).

Zinc has been known to be essential for the synthesis of nucleic acids and protein because impaired growth, protein synthesis, and deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) metabolism are primary features of zinc deficiency in animals (Li and Vallee, 1980). In zinc deficient suckling rats, growth was impaired and the activity of DNA-dependent RNA polymerase from the liver was decreased (Terhune and Sandstead, 1972). Zinc deficient rats showed low levels of DNA, RNA, collagen and non-collagenous protein synthesis in the connective tissue (Fernandes-Madrid et al., 1973). Hsu and Anthony (1973) reported that in zinc deficient rats, there was reduced collagen syn-

thesis and DNA synthesis in the skin.

Role of Zinc in Cell Biology: More details in the mechanism of zinc involvement in nucleic acid and protein synthesis have been revealed by studies using microorganisms. DNA polymerase (Slater et al., 1971; Springgate et al., 1973) and RNA polymerase (Scrutton et al., 1971) were isolated from Escherichia coli and shown to contain 2 gm atoms of bound zinc in one mole of each enzyme. Now these enzymes are believed to be zinc metalloenzymes (Li and Vallee, 1980). Rubin and Koide (1975) demonstrated zinc dependency for nucleic acid synthesis on the cellular level. Zinc reversed the inhibition of DNA synthesis in chick embryo cells induced by ethylene diamine tetraacetate (EDTA).

The cell cycle is the sequence of metabolic events in the mitotic process which produces two daughter cells, and consists of the G_1, S, G_2 phases and the period of mitosis, M (Cold Spring Harbor Laboratory, 1972). The G_1 phase is the post-mitotic phase in which RNA synthesis and protein synthesis occur. The S phase is the period of DNA synthesis, which is followed by the premitotic phase, G_2 and nuclear division (mitosis) (Falchuk et al., 1975). The role of zinc in the process of cell division was investigated using Euglena Gracilis by Falchuk et al. (1975). Zinc deficiency (1×10^{-7} M zinc) caused growth arrest; the size of cells increased while cellular RNA and protein content decreased, suggesting that zinc deficiency interferes with the progression of the cell cycle before the G_2 phase. These results were comparable to the results obtained by Wacker (1962). Zinc deficiency in Euglena Gracilis caused decreased cellular protein, decreased RNA content, and increased amino acid, polyphosphate, and DNA. These findings suggest that zinc is essential not only for protein and nucleic acid synthesis but also for cell division and differentiation (Li and Vallee, 1980).

The stimulatory effect of zinc on the growth of various kinds of cells has been studied by the following investigators. Zinc addition of 5×10^{-7} M (0.033 µg/ml) to a chemically defined medium produced

maximal cell growth of L mouse fibroblasts (Higuchi, 1970). Growth of Euglena Glacilis was enhanced by 1 x 10^{-5}M (0.65 μg/ml) zinc in the medium (Falchuk et al., 1975). Concentrations of from 1.5 to 4.5 x 10^{-4}M (9 to 29 μg/ml) of zinc in the medium had a stimulatory effect on mitosis of lymphocytes (Chvapil, 1976). An addition of 10 μM of $ZnCl_2$ (0.65 μg/ml) enhanced mitosis of hamster lymph node cells (Hart, 1978). Zinc concentrations from 0.1 to 0.2 mM (6.5 μg/ml to 13.1 μg/ml) stimulated DNA synthesis in lymphocytes (Chvapil et al., 1976a). Perfusion of 2 μ atoms zinc per hour in rat liver reversed inhibition of DNA synthesis by EDTA (Fujioka and Lieberman, 1964). The inhibition of thymidine incorporation by a chelator, o-phenanthroline, was reversed by the addition of 50 μM (3.27 μg/ml) zinc to human lymphocyte cultures (Williams and Loeb, 1973). When 0.6 μM (0.039 μg/ml) zinc was added to Dowex A-I resin-treated medium which contained zinc as low as 0.08 μM (0.006 μg/ml), maximal cell growth was attained in L cells (Thomas and Johnson, 1967). Thus, zinc has a beneficial effect on the growth of various cells.

However, zinc sometimes shows inhibitory and toxic effects on cellular functions, depending upon its concentration. Zinc inhibits electron transport in the mitochondrial respiratory chain at 2 μM (0.13 μg/ml) (Chvapil, 1973). Chvapil (1976) stated that addition of greater than 30.6 μM (2.00 μg/ml) of zinc to the culture medium inhibited the oxygen-consumption of activated leukocytes. The activity of ATPase was inhibited by a zinc concentration of 0.5 mM (33 μg/ml) in activated alveolar macrophages (Chvapil, 1976).

Zinc Effect on Biomembrane: There are several proposed mechanisms for the effect of zinc on biomembranes by Chvapil (1976) and also by Bettger and O'Dell (1981). The information is summarized as follows:

1. Zinc changes the fluidity of the membrane through interaction with some functional groups on the plasma membrane such as thiol groups (Warren et al., 1966) or the carboxy groups of protein (Perkins, 1964), or possibly by linking to

a phosphate moiety of phospholipids.

2. Zinc alters the integrity of the membrane structure through immobilization of energy dependent activities of the membrane by inhibition of ATPase (Stankova et al., 1976) or phospholipase A_2 (Wells, 1973).
3. Zinc protects a variety of membrane systems against peroxidative damage. An initiation step of H_2O_2 production, nicotinamide adenine dinucleotide phosphate (NADPH) oxidation, is inhibited by zinc in liver microsomes (Chvapil et al., 1976b) and in the granular fraction of rabbit lung alveolar macrophages (Stankova et al., 1976). A superoxide dismutase has been shown to be a zinc metalloenzyme and to play a key role in the protection of tissues against the toxic effect of superoxide anion (Fee and Teitelbaum, 1972).

MATERIALS AND METHODS

Cells

Normal skin fibroblasts (passage 2, ATCC No. CCD-48SK) were obtained from the American Type Culture Collection (Rockville, MD). The cells were utilized between the 6th and 13th passage. Stock cell cultures were maintained in a humidified incubator (5% CO_2) at 37°C in 75 cm^2 tissue culture flasks (Corning, Palo Alto, CA) containing 15 ml of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), and 1% (v/v) penicillin-streptomycin solution (100 units/ml : 50,000 units/500 ml respectively). The DMEM, FCS, and penicillin-streptomycin solution were purchased from Grand Island Biological Co. (GIBCO, Grand Island, NY). Some of the stock cells with early passages were frozen for storage and thawed to restart the culture. Therefore, it was possible to use cells of early passages (6 through 13) in all the experiments.

Zinc Removal from FCS and DMEM

Iminodiacetate (iminodiacetic acid, disodium salt) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Epoxy-activated Sepharose 6B was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Iminodiacetate was coupled to epoxy-activated Sepharose 6B according to the manufacture's directions (Pharmacia Fine Chemicals, 1979). Selective extraction of zinc and copper from FCS and DMEM was carried out as described by Messer et al. (1982).

The gel coupled with iminodiacetate was equilibrated with the major cations other than zinc and copper. The equilibrating solution was prepared from major salts present in the DMEM. The equilibrating solution contained (in mg/l); $CaCl_2 \cdot 2H_2O$, 265; $MgSO_4 \cdot 7H_2O$, 200; KCl, 400; NaCl, 6400; $NaHCO_3$, 3700; $NaH_2PO_4 \cdot H_2O$, 125. For the extraction of serum, a salt solution was prepared consisting of $CaCl_2 \cdot 2H_2O$, 491;

$MgCl_2 \cdot 6H_2O$, 244; NaCl, 9000; KH_2PO_4, 60; KCl, 400 mg/l. The gel was added in the proportion of 1 ml gel to 100 ml solution to the equilibrating solution and held overnight. The gel was washed once with deionized water and suction-dried before being added to the DMEM or FCS. The resulting gel was added to DMEM in the proportion of 1 ml gel to 50 ml DMEM and left at room temperature for 2 hours. After the gel was sedimented and removed, the medium was sterilized by filtering through 0.20 micron filter (Nalge, Rochester, NY). The gel was added to FCS in the proportion of 2 ml gel to 10 ml FCS and incubated for 1 hour at room temperature. After the incubation, the gel was removed by centrifugation and the extraction was repeated once with the gel and sterilized by filtration using 0.45 micron filter (millipore, San Francisco, CA). The levels of zinc, copper, calcium, magnesium, and manganese in media and serum were analyzed before and after the extraction by atomic absorption spectrometry without any manipulation except for dilution of the samples with water. Analysis of the minerals were performed in the Department of Soil Science of Oregon State University.

The Effect of Zinc Supplementation of the Medium on the Growth of Human Fibroblasts.

With Intact Medium (Study I and II): Cells in stock flasks were dissociated with 0.05% trypsin-0.02% EDTA and were seeded on day 0 at a concentration of 5×10^4 cells per dish into 60 mm tissue culture dishes (Corning) containing 3 ml of medium consisting of DMEM, 10% (v/v) FCS, and 1% (v/v) penicillin-streptomycin solution. The next day the medium was replaced with 5 ml of 5% (v/v) FCS containing medium supplemented with various levels of zinc. Zinc stock solution was made of zinc sulfate ($ZnSO_4 \cdot 7H_2O$)(Mallinckodt, St. Louis,MO) and deionized water. Various amounts of the stock solution were added to the growth medium to make the concentration of added zinc in the medium range from 0 μg/ml to 10 μg/ml. The growth medium was changed twice during the 7 day incubation. On the 7th day of incubation, the cells were detached in 2 ml of trypsin-EDTA solution and

centrifuged at 1000 rpm for 5 minutes after addition of 4 ml of Dulbecco's phosphate buffered saline (DPBS). Cells were resuspended with 2 ml of DPBS and the number of cells were counted by use of a hemocytometer. Protein content of the cells was determined by the Lowry method (Lowry et al., 1951) after repeated freezing and thawing of the cell suspension.

With Zinc Extracted Medium (Study III): The experimental design was the same as in the above study except for the following changes: on day 1, the intact growth medium containing 10% (v/v) FCS was replaced with 3 ml of zinc extracted medium (5% FCS) with various amounts of exogenous zinc as for the studies I and II. The growth medium was changed three times during the 9 days of incubation.

LDL Binding and Degradation Study

Lipoprotein Isolation: Human LDL (density 1.006 to 1.063 g/ml) and lipoprotein deficient serum (density 1.215 g/ml) were obtained from 150 ml of blood collected in Vacutainers containing 0.01% EDTA from healthy subjects who had been fasted over 15 hours. Lipoproteins were prepared by differential ultracentrifugation according to Havel et al. (1955). Isolated fractions were extensively dialyzed for at least 30 hours at 4°C against buffer containing 0.15 M NaCl and 0.3 mM EDTA, at pH 7.4. Following dialysis, the lipoprotein deficient serum was defibrinated as described by Brown et al. (1974). The lipoprotein deficient serum was incubated at 24°C for 10 minutes in the presence of thrombin (20 units per ml)(Cat. No. T-9000, Sigma) and the clot was removed by a glass rod. To obtain clear serum, the serum was centrifuged at 30,000 x g for 2 hours at 4°C. The dialyzed LDL and defibrinated lipoprotein deficient serum were sterilized by filtration through a 0.22 micron Millipore filter and stored at 4°C. Total cholesterol content of lipoprotein fractions was determined by the enzymatic method described by Allain et al. (1974). The LDL

fraction contained 4.67 ± 0.02 mg/ml total cholesterol. However, the amount of total cholesterol in the lipoprotein deficient fraction was too small to be determined by this method. Protein concentration in the LDL fraction (3.5 mg/ml) was determined by the Lowry method (Lowry et al., 1951).

Preparation of ^{125}I-LDL: LDL iodination was performed as described by McFarlane (1958). 750 µg/ml of 1 M glycin buffer at pH 10, 10 µg of sodium iodide (^{125}I) in NaOH solution (100 mCi/ml, Lot No. 37BA, Amersham International Ltd., Arlington Heights, IL), and 350 µl of iodine monochloride solution (Aldrich Chemical Co.) were added sequentially to 1.5 ml (contained 5.25 mg LDL protein) of dialyzed LDL solution, voltexing at each addition. Free iodine was removed by extensive dialysis against buffer containing 0.1 M KI, 0.15 M NaCl, and 0.01% EDTA, pH 7.0 over 35 hours at 4°C. The protein content in the final ^{125}I-LDL fraction was determined as 1.7 mg/ml by the Lowry method (Lowry et al., 1951). The final specific activity of the ^{125}I-LDL solution was 101-counts per minutes (cpm) per ng of protein. A Packard Gamma Counter (Model 5230) was used to count radioactivity. The ^{125}I-LDL was stored at 4°C and it was used within 3 days for the LDL binding experiment.

Preparation of Cells Prior to the LDL Binding Study: On day 0, human fibroblast cells were prepared from the stock flasks described earlier and seeded at a concentration of 3.5×10^5 cells per dish into 60-60 mm tissue culture dishes containing 3 ml of medium consisting of 10% (v/v) FCS and incubated in a humidified incubator (5% CO_2) at 37°C. The next day the dishes were divided into 4 groups for the 4 different zinc levels. The medium was replaced with 3 ml of zinc extracted medium (5% FCS) which was supplemented with 4 levels of exogenous zinc. The concentrations of exogenous zinc in the media were 0, 1.5, 4.0, and 8.0 µg/ml. The growth medium with or without exogenous zinc was changed with fresh medium every other day. On the 8th day of incubation, the medium was replaced with 3 ml of medium

consisting of zinc extracted DMEM with 10% (v/v) lipoprotein deficient serum, 1% (v/v) penicillin-streptomycin solution. There were 4 levels of zinc supplementations as described above. All culture dishes became nearly confluent before the 9th day of incubation.

Incubation of Cells in ^{125}I-LDL Containing Medium: After 24 hours of incubation in the medium containing lipoprotein deficient serum, the medium was removed and cells were washed once with 5 ml of zinc extracted DMEM. Then 15 dishes for each zinc level received 3 ml of medium containing zinc extracted DMEM and 10% (v/v) lipoprotein deficient serum, 5 μg/ml of human ^{125}I-LDL protein (101 cpm/ng of protein), and unlabeled lipoprotein at concentrations of 0, 10, 25, 50, and 100 μg/ml. All cells were incubated for 5 hours in a humidified incubator (5% CO_2) at 37°C.

Determination of ^{125}I-LDL Binding and Internalization in the Cells: After 5 hours of incubation the medium was removed and all subsequent operations were carried out at 4°C (on an ice sheet). Cell monolayers were washed three times with 2 ml of ice cold Buffer A containing 2.0 g bovine serum albumin, 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.2 g KH_2PO_4 per liter. Then the cell monolayers were incubated for 2 minutes with 2 ml of the same buffer on an ice sheet. This latter step was repeated once. Afterward, each cell monolayer was washed once with Buffer A without albumin and received 1 ml of 4N NaOH. Within 30 minutes all cells were dissolved. All cell aliquotes were transfered to Lowry tubes. Aliquots of 500 μl were used for the measurement of radioactivity by a gamma counter and then frozen 30 days until they were used for the determination of cellular cholesterol. Each dish contained 330 to 500 μg of total cell protein. Counted radioactivity (cpm) was converted to ng of ^{125}I-LDL protein or total LDL protein and the results were expressed per mg cell protein.

Determination of ^{125}I-LDL Degradation by the Cells (determination of acid soluble material): Bound ^{125}I-LDL protein is known to be hydrolyzed by the lysosomal enzymes and degraded to a product which is soluble in trichloroacetic acid (TCA), whereas free ^{125}I-LDL precipitates with the acid (Goldstein and Brown, 1974).

After incubation for the LDL binding, the medium was removed from dishes and mixed with 0.5 ml 50% (w/v) TCA and then incubated for 30 minutes on ice to precipitate unhydrolyzed proteins. To remove the precipitate, the entire solution was centrifuged for 10 minutes at 3000 rpm. One ml of supernatant was mixed with 10 µl of 40% KI and 40 µl of 30% H_2O_2 and incubated at room temperature for 5 minutes. The mixture was mixed with 2 ml $CHCl_3$ using a vortex and centrifuged for 5 minutes at 2500 rpm to remove free iodine which had been presented in the medium before the LDL binding. 500 µl of aqueous phase was taken to a counting tube for determination of radioactivity of iodine associated with tyrosine.

Determination of Total Cholesterol in Cells: Cell monolayers were dissolved with the 4N NaOH treatment; therefore, it was impossible to determine accurately the content of cholesterol ester after the LDL binding because some cholesterol ester may have been hydrolyzed by the strong base in the solution. Only total cholesterol content in the cells was determined.

500 µl of the dissolved cell aliquots were mixed with an internal standard (stigmasterol solution: 10 µg/20 µl) and 1 ml methanol, and incubated for 30 minutes at 80°C. Lipids were extracted from the mixture 4 times with 1 ml petroleum ether. After evaporation of the solvent, the extracted lipids were dissolved in 100 µl ethylacetate. A gas chromatograph (Hewlett Packard, 5710A) was used to determine cholesterol levels in the sample. A glass column was packed with 3% OV-17 (Supelco, Inc., Bellefonte, PA), and nitrogen was used as the carrier gas at a flow rate of about 40 ml/min. The oven temperature was 270°C. Both detector and injector temperature were 300°C.

Two µl of external standard containing 0.2 µg stigmasterol and 0.2 µg cholesterol were injected before 20 µl of a sample was injected into the column. Both cholesterol and stigmasterol peaks appeared within 15 minutes.

Statistics

Using a statistical program (SIPS), regression analysis and multiple comparison were performed for the data from the cell growth experiments. Factorial analysis was conducted on the data of the LDL binding experiments.

RESULTS

The Effect of Zinc Supplementation of the Intact Medium on the Growth of Human Fibroblasts. (Study I and II)

The results of cell growth study I are shown in figure 1 and 2. There was a significant negative correlation between the amount of zinc added and the number of cells in the dishes ($r = -0.88$, $p<0.01$). Zinc addition of 2 μg/ml to the media seemed to increase the number of cells and further increase of zinc lowered the number of cells, but statistical analysis showed no difference in mean number of cells when cells were grown in the medium supplemented with less than 4 μg/ml. This indicated that the amount of zinc present in the control dishes (which was later found as 0.210 μg/ml) was sufficient to support normal cell growth. Further increases of zinc supplementation above 4 μg/ml substantially decreased the mean number of cells. A similar effect of zinc was observed when the protein content of the cells was determined (see figure 2). There was a significant negative correlation between the amount of zinc added and the protein content in the cells ($r = -0.90$, $p<0.01$). As expected, the correlation between the number of cells in the dishes and the protein content of the cells was significant ($p<0.05$). As shown in figures 3 and 4, the above observations were confirmed when the experiment was repeated.

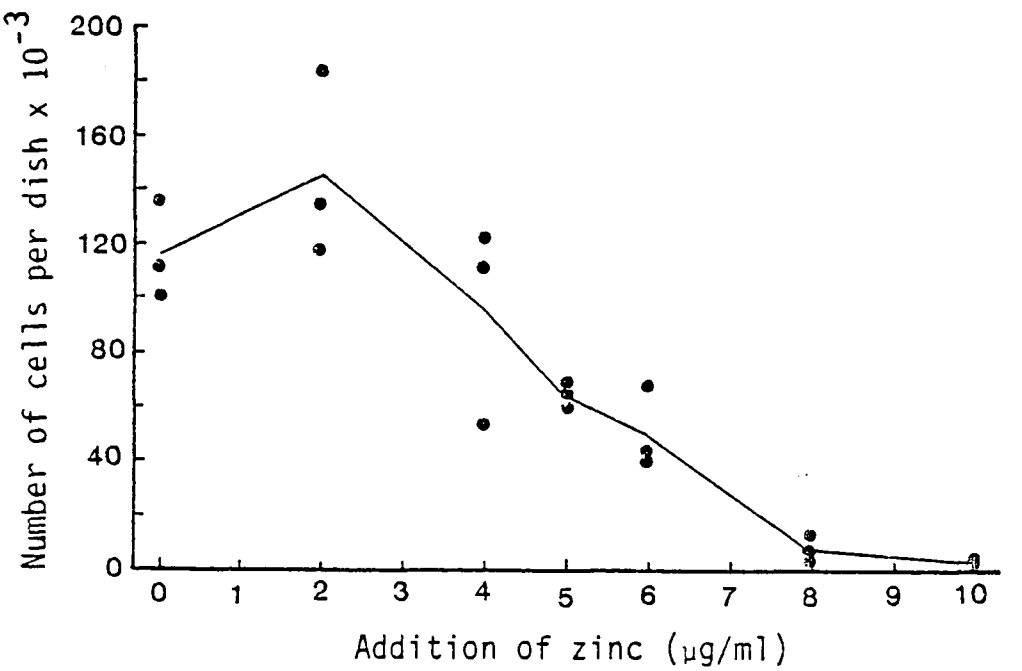

Figure 1. Study I. The number of cells per dish after 7 days of incubation in the medium supplemented with various zinc concentrations. The data points represent number of cells in each dish and the line is the mean of the value of three dishes.

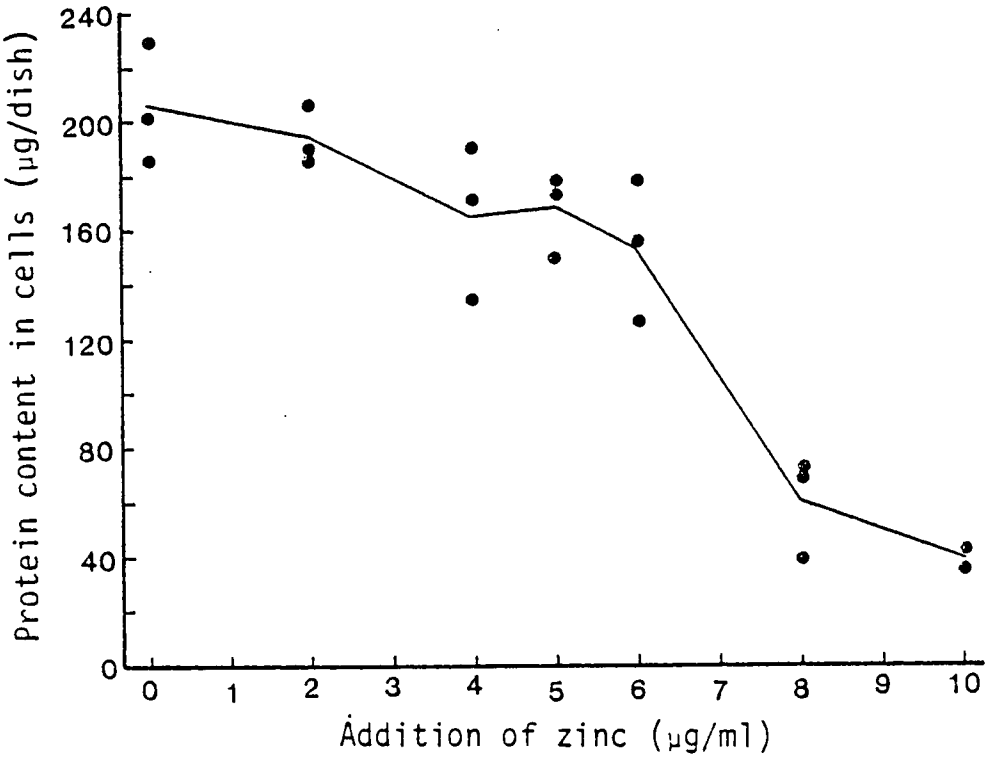

Figure 2. Study I. The protein content of cells after 7 days of incubation in the medium supplemented with various zinc concentrations. The data points represent number of cells in each dish and the line is the mean of the value of three dishes.

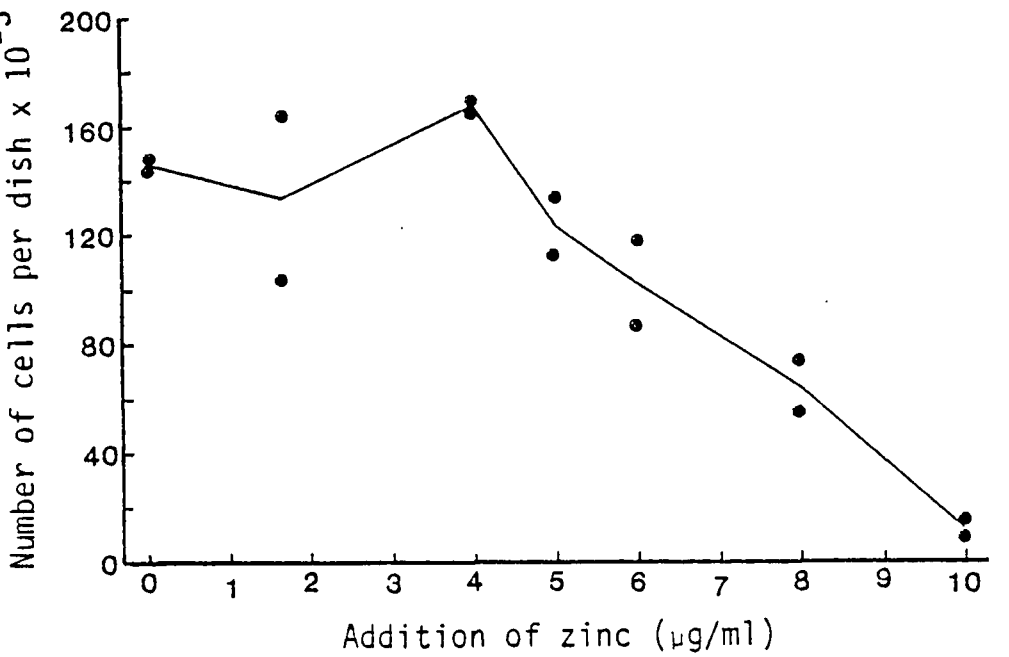

Figure 3. Study II. The number of cells per dish after 7 days of incubation in the medium supplemented with various zinc concentrations. The data points represent number of cells in each dish and the line is the mean of the value of two dishes.

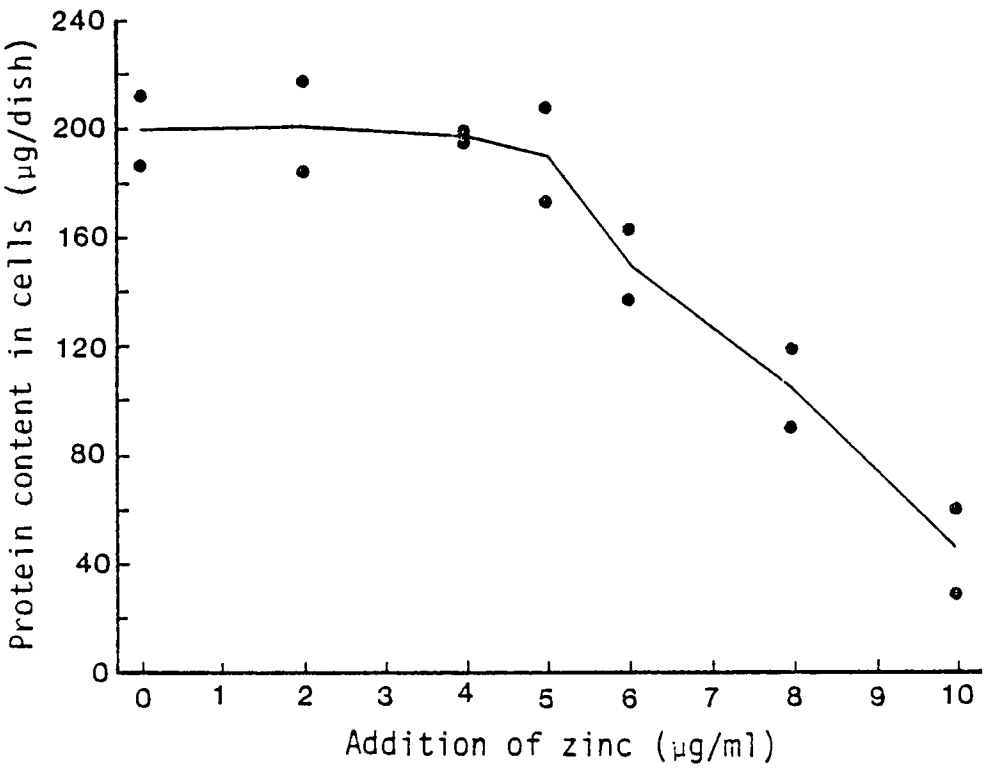

Figure 4. Study II. The protein content of cells after 7 days of incubation in the medium supplemented with various zinc concentrations. The data points represent number of cells in each dish and the line is the mean of the value of two dishes.

Removal of Zinc from Fetal Calf Serum(FCS) and Dulbecco's Modified Eagle Medium (DMEM)

To obtain zinc deficient medium, FCS and DMEM were subjected to the selective extraction of divalent cations as described by Messer (1982). The effect of extraction on zinc and other cation concentrations in FCS and DMEM is shown in tables 2, 3, and 4. From these data the amounts of metals in the medium containing 5% FCS before and after the extraction were calculated as shown in tables 5 and 6.

The extraction of divalent cations from FCS were carried out in two different sources of FCS: control lot number 27K5501 and 29D112. The effect of the extraction on the divalent cation composition of FCS (control lot number 27K5501) is shown in table 2. The magnesium content was not affected by the extraction procedure. Almost 90% of calcium and zinc, 62% of manganese, and 53% of copper were removed by the treatment. Table 3 shows the results of the trace metal extraction of FCS (control lot number 29D112). The treatment removed 12% of the magnesium, 69% of the calcium, 100% of the manganese, 60% of the copper and 88% of the zinc of the FCS.

Table 4 shows a summary of the metal content of DMEM after the extraction. Magnesium and manganese concentrations were unaffected by the treatment, whereas calcium concentrations were slightly increased (4%) by the treatment. The treatment removed 17% of the copper and 63% of the zinc from the DMEM.

The metal compositions in the medium containing 5% FCS as shown in tables 5 and 6 were calculated according to the data in tables 2, 3, and 4. Table 5 lists the results obtained from a control lot number 27K5501 FCS and DMEM. Although the magnesium content and calcium content were not changed very much by the extraction, 35% of manganese, 25% of the copper, and 79% of the zinc were removed by the treatment. Zinc concentration was reduced from 0.210 µg/ml to 0.045 µg/ml. Appropriate amounts of manganese and copper were supplemented to the extracted medium containing 5% FCS in order to reconstitute

original concentrations so that the final medium used for cell culture was deficient in zinc only. This zinc deficient medium was used for cell growth study III, the results of which are shown in figures 5 and 6.

Table 6 shows the calculated values of divalent cations in the 5% FCS containing medium from the control lot number 29D112 FCS and DMEM. Changes in magnesium and calcium concentrations before and after the extraction were very small. The extraction removed 12% of the manganese, 8% of the copper, and 81% of the zinc. Supplementatation of manganese and copper in the extracted medium was not essential in this case because the loss of these metals by extraction was very small. This medium was used for the lipoprotein binding study.

Table 2. Effect of extraction on metal composition of FCS[1].

	Unextracted (μg/ml)	Extracted (μg/ml)	Percent Reduction* (%)
Magnesium($x100^{-1}$)	0.23 ± 0.00	0.22 ± 0.01	4
Calcium($x100^{-1}$)	1.37 ± 0.01	0.15 ± 0.01	89
Manganese	0.13 ± 0.01	0.05 ± 0.01	62
Copper	0.32 ± 0.01	0.15 ± 0.01	53
Zinc	2.67 ± 0.01	0.33 ± 0.01	88

[1]Serum (control lot number 27K5501) was extracted twice with iminodiacetate gel (2 ml gel to 10 ml serum). The numbers are means and standard deviations of three measurements by atomic absorption spectrometry.
*Percentage of metals removed by the extraction.

Table 3. Effect of extraction on metal composition of FCS[1].

	Unextracted (μg/ml)	Extracted (μg/ml)	Percent Reduction (%)
Magnesium($x100^{-1}$)	0.34 ± 0.01	0.30 ± 0.00	12
Calcium($x100^{-1}$)	1.37 ± 0.01	0.43 ± 0.01	69
Manganese	0.08 ± 0.00	0.00 ± 0.00	100
Copper	0.30 ± 0.01	0.12 ± 0.01	60
Zinc	4.10 ± 0.01	0.50 ± 0.01	88

[1]Serum (control lot number 29D112) was extracted twice with iminodiacetate gel (2 ml gel to 10 ml serum). The numbers are means and standard deviations of three measurements by atomic absorption spectrometry.

Table 4. Effect of extraction on metal composition of DMEM.

	Unextracted (μg/ml)	Extracted (μg/ml)	Percent Reduction (%)
Magnesium($x100^{-1}$)	0.21 ± 0.00	0.21 ± 0.00	0
Calcium($x100^{-1}$)	0.70 ± 0.01	0.73 ± 0.00	-4
Manganese	0.03 ± 0.00	0.03 ± 0.00	0
Copper	0.06 ± 0.01	0.05 ± 0.01	17
Zinc	0.08 ± 0.00	0.03 ± 0.01	63

DMEM was extracted once with iminodiacetate gel (1 ml gel to 50 ml media). The numbers are means and standard deviations of three measurements by atomic absorption spectrometry. This extracted DMEM was used for cell growth study and LDL binding study.

Table 5. Effect of extraction on metal composition of medium supplemented with 5% (v/v) FCS[1].

	Unextracted (μg/ml)	Extracted (μg/ml)	Percent Reduction (%)
Magnesium($x100^{-1}$)	0.210	0.210	0
Calcium($x100^{-1}$)	0.734	0.701	4
Manganese	0.048	0.031	35
Copper	0.073	0.055	25
Zinc	0.210	0.045	79

[1]Metal compositions were calculated from FCS(in table 2) and DMEM (in table 4). This mixture of FCS and DMEM was used for cell growth study III after manganese and copper were added to the medium to reconstitute original concentrations.

Table 6. Effect of extraction on metal composition of medium supplemented with 5% (v/v) FCS[1].

	Unextracted (μg/ml)	Extracted (μg/ml)	Percent Reduction (%)
Magnesium($x100^{-1}$)	0.217	0.215	1
Calcium($x100^{-1}$)	0.734	0.715	3
Manganese	0.033	0.029	12
Copper	0.059	0.054	8
Zinc	0.281	0.054	81

[1]Metal compositions were calculated from FCS(in table 3) and DMEM (in table 4). This mixture of FCS and DMEM was used for the cell growth period of LDL binding experiment.

The Effect of Zinc Supplementation of the Zinc Extracted Medium on the Growth of Human Fibroblasts. (Study III)

There was no difference in either the number of cells or protein content in the cells when up to 6 μg/ml zinc was added to the zinc extracted culture medium; however, further addition of zinc to the medium caused a significant decrease in both the number of cells and the cell protein content ($p<0.01$)(Figures 5 and 6). The trend of the decrease in the number of cells was moderate compared with the earlier results obtained from studies I and II which did not use a zinc extracted medium. As shown in studies I and II, the correlation between the number of cells and the protein content of cells was significant ($p<0.01$).

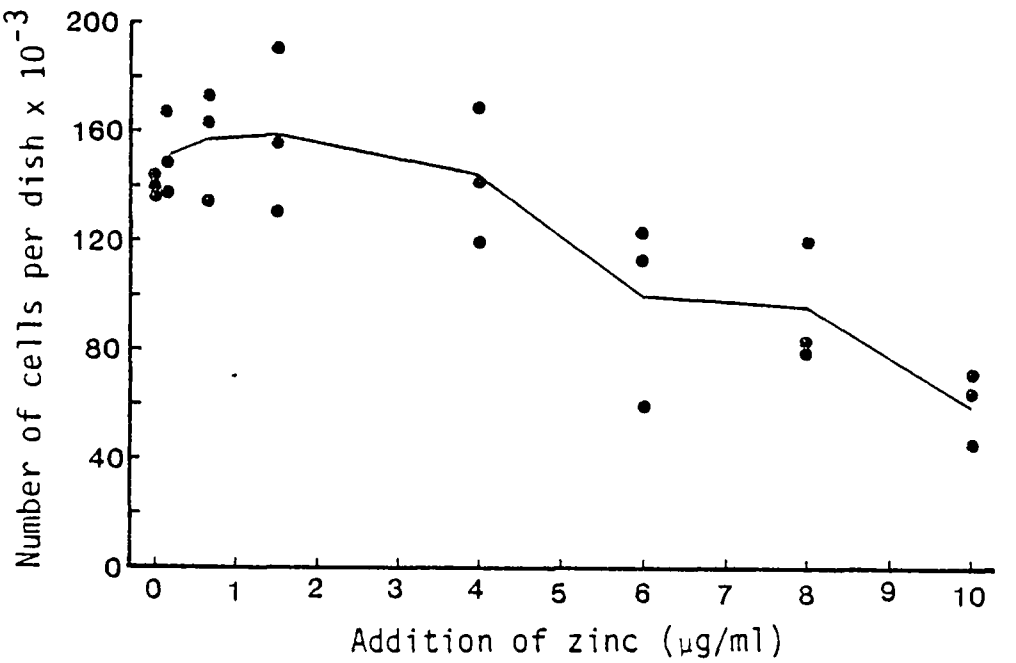

Figure 5. Study III. The number of cells per dish after 9 days of incubation in the medium supplemented with various zinc concentrations. The data points represent number of cells in each dish and the line is the mean of the value of three dishes.

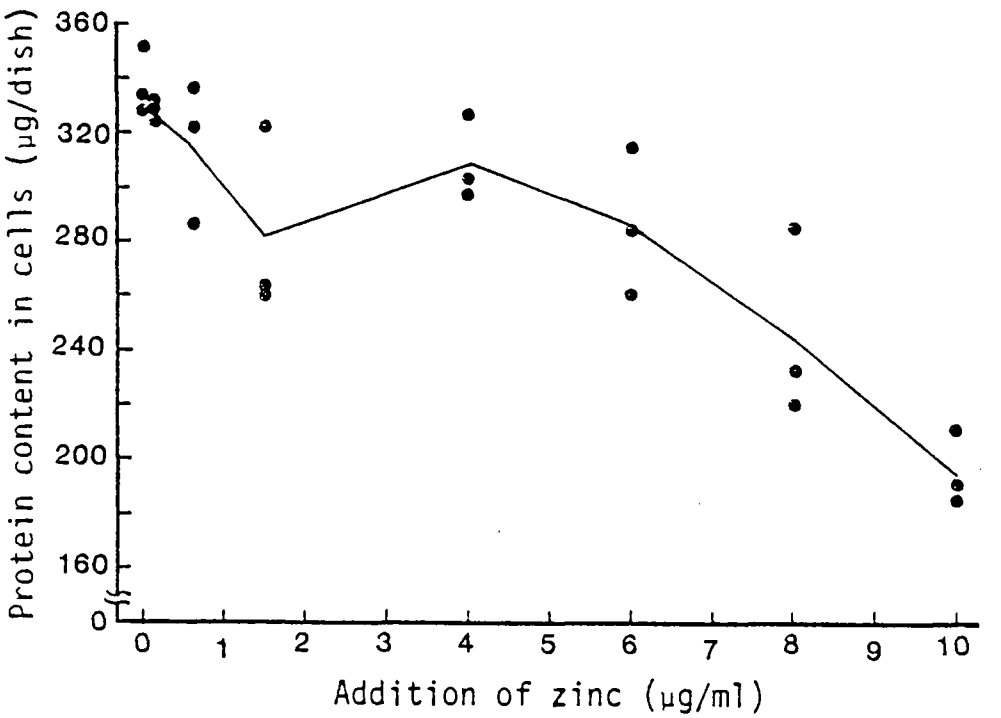

Figure 6. Study III. The protein content of cells after 9 days of incubation in the medium supplemented with various zinc concentrations. The data points represent protein content of cells in each dish and a line is the mean of the value of three dishes.

LDL Binding and Degradation Study

Human fibroblasts were plated in each dish (3.5×10^5/dish) on day 0. Zinc-removed medium containing 5% FCS with 4 levels of exogenous zinc was changed every other day during the cell growth period. On day 8, the medium was replaced with medium containing lipoprotein deficient serum, and on day 9 the LDL binding study was carried out. The medium used for the LDL binding study contained 5 μg/ml of ^{125}I-LDL and 5 levels of unlabeled LDL (0, 10, 25, 50, and 100 μg/ml). Triplicate dishes were used for each level of LDL. After 5 hours of incubation of cells at 37°C, the medium was removed for the measurement of acid soluble material. Cells were washed and harvested to count radioactivity and to measure cell protein and cholesterol content.

Figures 7 and 8 show the relationships between the amount of LDL bound to the cells and the level of unlabeled LDL added to the medium. Addition of unlabeled LDL to the medium significantly increased the amount of LDL bound to the cells ($p<0.01$). Addition of zinc to the growth medium (1.5 μg/ml) significantly increased the cellular LDL level compared with the controls ($p<0.05$) except when 50 μg/ml unlabeled LDL was added to the medium (Figure 8). Addition of zinc at greater than 1.5 μg/ml to the growth medium tended to suppress LDL binding. This effect was statistically significant in the cultures receiving unlabeled LDL of 0, 25, and 100 μg/ml ($p<0.05$). Table 7 lists the means and the standard deviations of the results shown in figures 7 and 8.

Figures 9 and 10 illustrate the relationship between the amount of LDL degraded by the cells and the level of unlabeled LDL added to the medium. Addition of unlabeled LDL to the medium significantly increased the amount of LDL degraded by the cells ($p<0.01$). Compared with the LDL binding, zinc had little effect on the LDL degradation. Without addition of unlabeled LDL, zinc addition (1.5 μg/ml) to the medium significantly increased the mean LDL degraded by the cell ($p<0.05$). When unlabeled LDL (25 μg/ml) was added to the medium,

the mean LDL degraded by the cells grown in the presence of 1.5 μg/ml or 4.0 μg/ml of zinc was significantly higher than that degraded by the cells grown in the presence of 8 μg/ml zinc($p<0.05$). With unlabeled LDL (100 μg/ml) in the medium, zinc addition (4.0 μg/ml) to the medium significantly increased the mean LDL degraded by the cell($p< 0.05$).

The amount of ^{125}I-LDL bound and internalized to the cells was significantly reduced by the addition of unlabeled LDL to the medium ($p<0.01$)(Figure 11). The amount of ^{125}I-LDL bound and internalized into cells grown in presence of 1.5 μg/ml zinc was significantly increased compared with the controls($p<0.01$), except when 50 μg/ml of unlabeled LDL was added to the medium(Figure 12). Zinc addition to the growth medium of greater than 1.5 μg/ml tended to suppress ^{125}I-LDL binding. This effect was statistically significant for cultures receiving unlabeled LDL at 0, 25, and 100 μg/ml ($p<0.05$). Table 9 shows the means and the standard deviations of the data presented in figures 11 and 12.

The amount of ^{125}I-LDL degraded by the cells was significantly reduced by the addition of unlabeled LDL to the medium ($p<0.01$) (see figure 13). Again, zinc had little effect on the amount of ^{125}I-LDL degraded by the cells. Addition of zinc (1.5 μg/ml) to the growth medium did not cause any significant increase or decrease in the means of the amount of degraded ^{125}I-LDL compared to the controls (figure 14). Compared to the addition of zinc of 1.5 μg/ml, 8 μg/ml of zinc caused a significant decrease in the mean degraded ^{125}I-LDL value when cells had either 0 or 25 μg/ml of additional unlabeled LDL in the medium ($p< 0.01$). When 25 μg/ml or 100 μg/ml unlabeled LDL were added to the medium, the addition of zinc in amounts greater than 4 μg/ml to the growth medium significantly decreased the mean degraded ^{125}I-LDL value ($p<0.05$). The means and the standard deviations of the results shown in figures 13 and 14 are given in table 10.

Table 11 shows the cholesterol content in the cells after the LDL binding. There was no significant effect of zinc addition to the medium during the cell growth period on the cellular cholesterol content. Without zinc addition, cellular cholesterol levels were significantly higher than the controls with 50 and 100 μg/ml of added unlabeled LDL ($p < 0.01$). With the addition of 1.5 μg/ml of zinc to the growth medium, the mean cellular cholesterol levels with unlabeled LDL added at levels greater than 25 μg/ml were significantly higher than that at 10 μg/ml of unlabeled LDL ($p < 0.01$). With 4.0 μg/ml of zinc added to the growth medium, the addition of unlabeled LDL to the LDL binding medium caused a significant increase in the mean cell cholesterol levels as compared to the controls. With the highest zinc addition (8 μg/ml) to the growth medium, the mean cholesterol content in the cells which received over 50 μg/ml of unlabeled LDL in the LDL binding medium was significantly higher than that in the cells with 10 μg/ml of unlabeled LDL ($p < 0.01$).

Taken together, these results suggest that the addition of higher levels of unlabeled LDL to the medium enhances LDL binding which in turn brings about increased cholesterol levels in the cells.

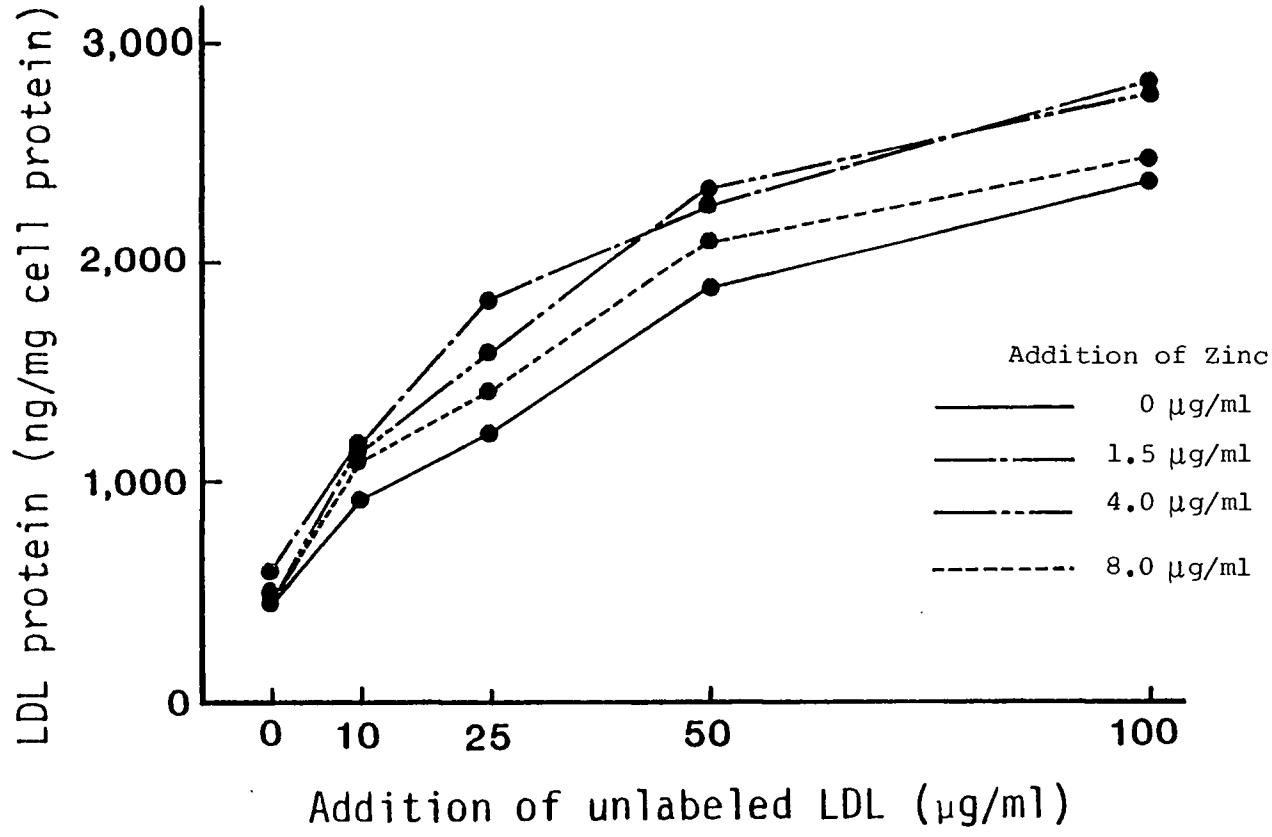

Figure 7. Bound and internalized LDL in normal human fibroblasts grown in various zinc concentrations. Each point represents the mean of three culture dishes. All dishes contained ^{125}I-LDL (5 μg/ml) in addition to the indicated amount of unlabeled LDL.

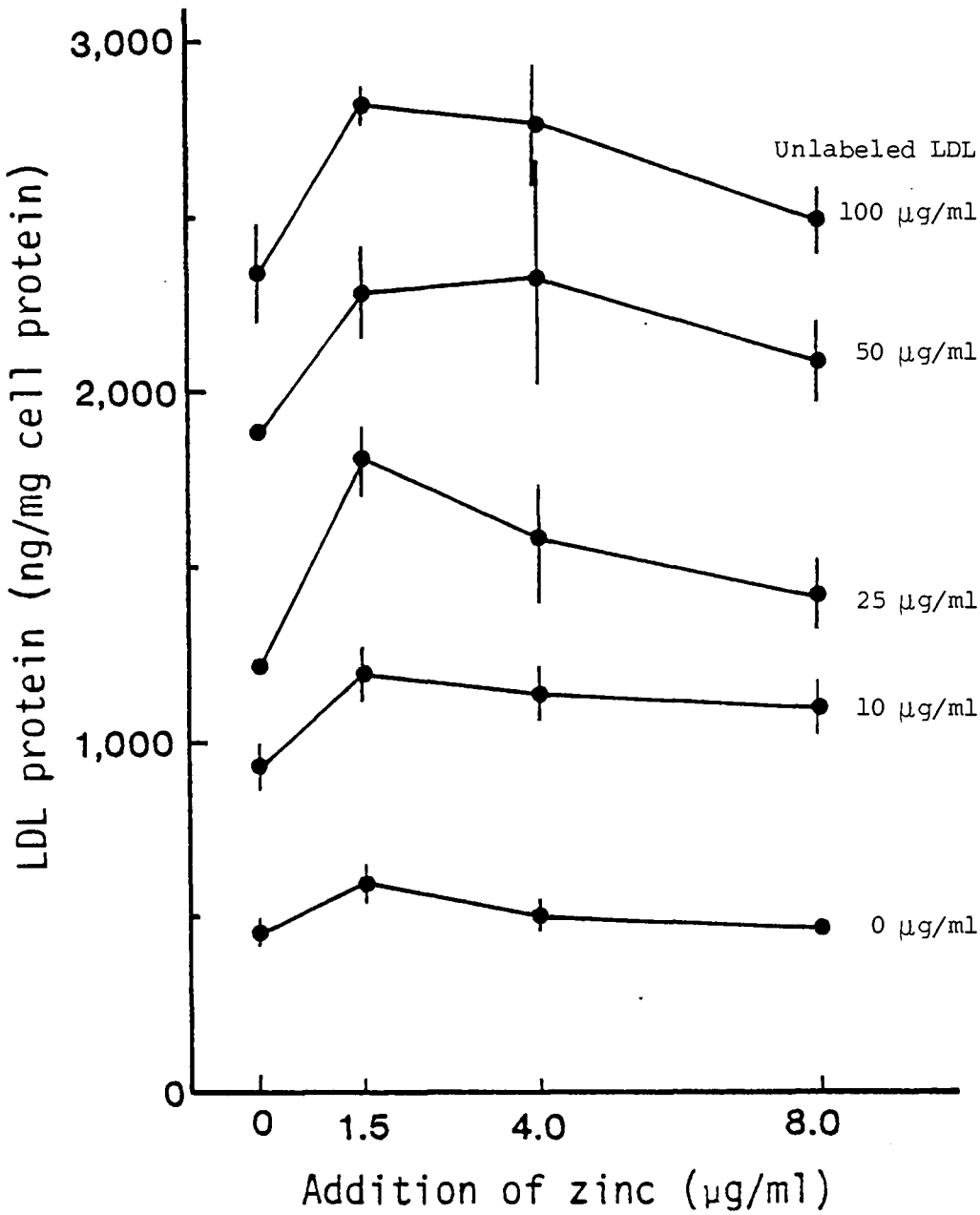

Figure 8. Bound and internalized LDL in normal human fibroblasts grown in various zinc concentrations. Each point represents the mean of the three culture dishes and standard deviation. All dishes contained ^{125}I-LDL (5 µg/ml) in addition to the indicated amount of unlabeled LDL.

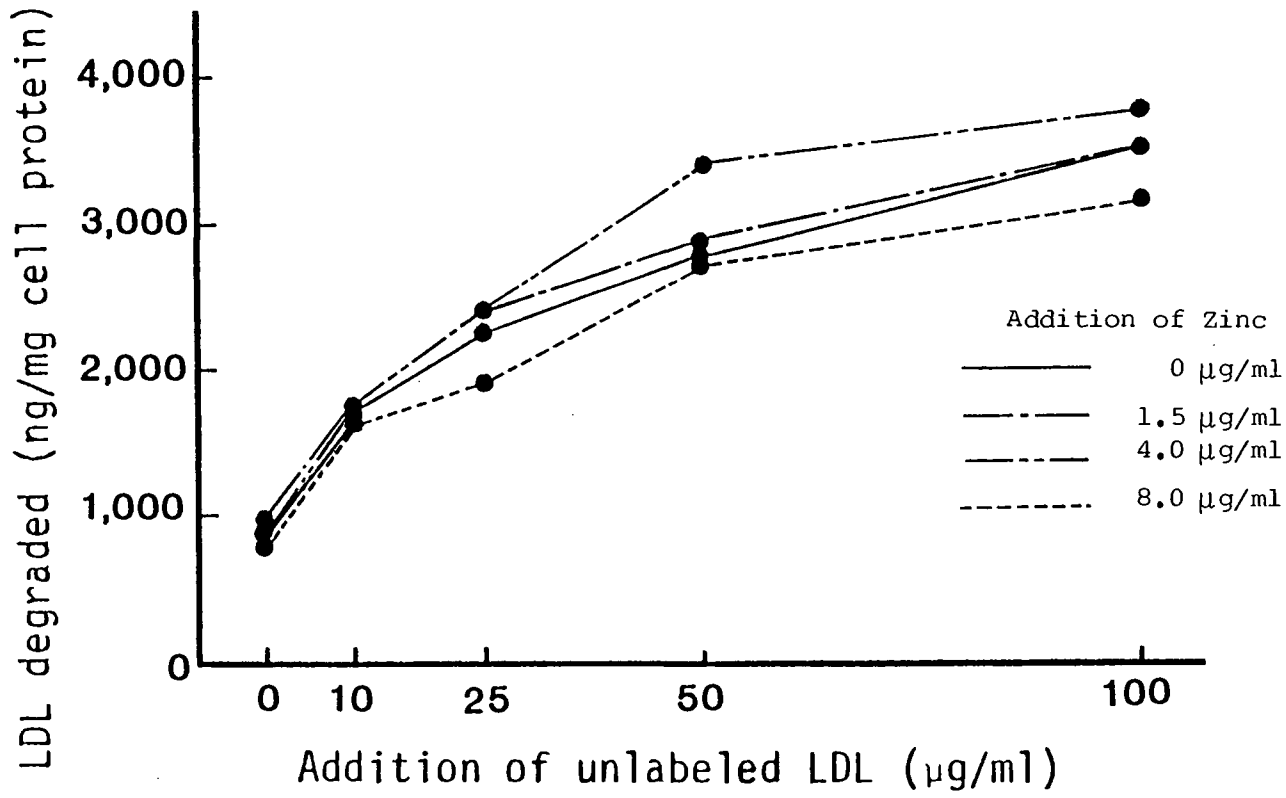

Figure 9. Degradation of LDL by normal human fibroblasts grown in various zinc concentrations. Each point represents the mean of the three culture dishes. All dishes contained ^{125}I-LDL (5 μg/ml) in addition to the indicated amount of unlabeled LDL.

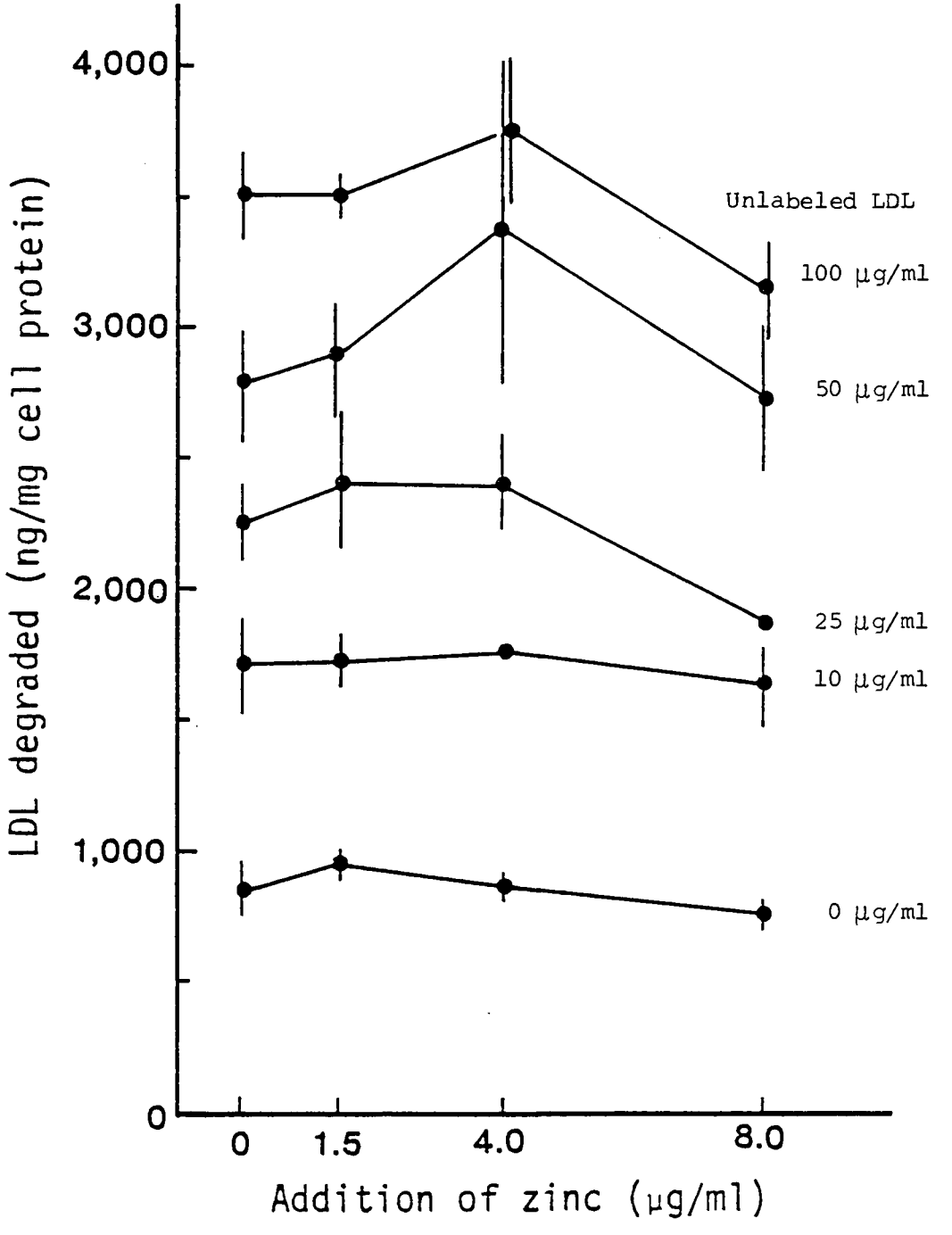

Figure 10. Degradation of LDL by normal human fibroblasts grown in various zinc concentrations. Each point represents the mean of the three culture dishes. All dishes contained ^{125}I-LDL (5 µg/ml) in addition to the indicated amount of unlabeled LDL.

Table 7. Means of bound and internalized LDL in normal human fibroblasts (LDL protein ng/mg cell protein).

Addition of Zinc (μg/ml)	Addition of Unlabeled LDL(μg/ml)				
	0	10	25	50	100
0	457 ± 31	933 ± 71	1210 ± 29	1881 ± 194	2338 ± 133
1.5	598 ± 23	1195 ± 81	1802 ± 98	2284 ± 131	2828 ± 44
4.0	499 ± 42	1124 ± 64	1568 ± 172	2339 ± 327	2772 ± 187
8.0	460 ± 13	1084 ± 79	1410 ± 98	2094 ± 121	2471 ± 85

Each dish contained 5 μg/ml ^{125}I-LDL in medium in addition to the indicated amount of unlabeled LDL.

Table 8. Means of degraded LDL by normal human fibroblasts(LDL protein ng/mg cell protein).

Addition of Zinc (μg/ml)	Addition of Unlabeled LDL(μg/ml)				
	0	10	25	50	100
0	837 ± 97	1703 ± 146	2238 ± 140	2776 ± 194	3514 ± 175
1.5	956 ± 48	1704 ± 103	2408 ± 250	2889 ± 253	3507 ± 73
4.0	869 ± 49	1763 ± 44	2390 ± 229	3406 ± 613	3766 ± 291
8.0	756 ± 40	1629 ± 131	1872 ± 6	2717 ± 264	3143 ± 175

Each dish contained 5 μg/ml ^{125}I-LDL in medium in addition to the indicated amount of unlabeled LDL.

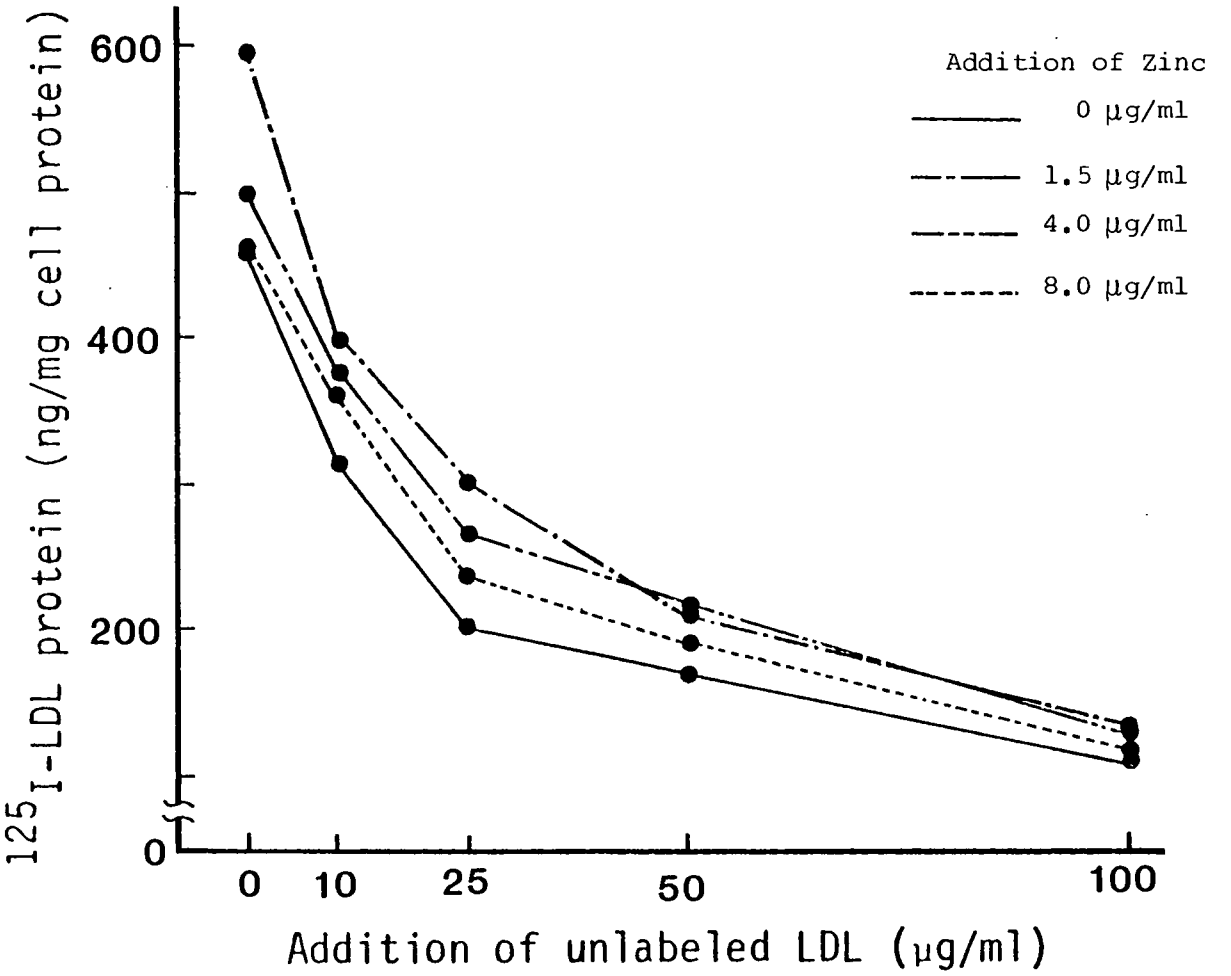

Figure 11. Bound and internalized ^{125}I-LDL to normal human fibroblasts grown in various zinc concentrations. Each point represents the mean of the three culture dishes. All dishes contained ^{125}I-LDL (5 µg/ml) in addition to the indicated amount of unlabeled LDL.

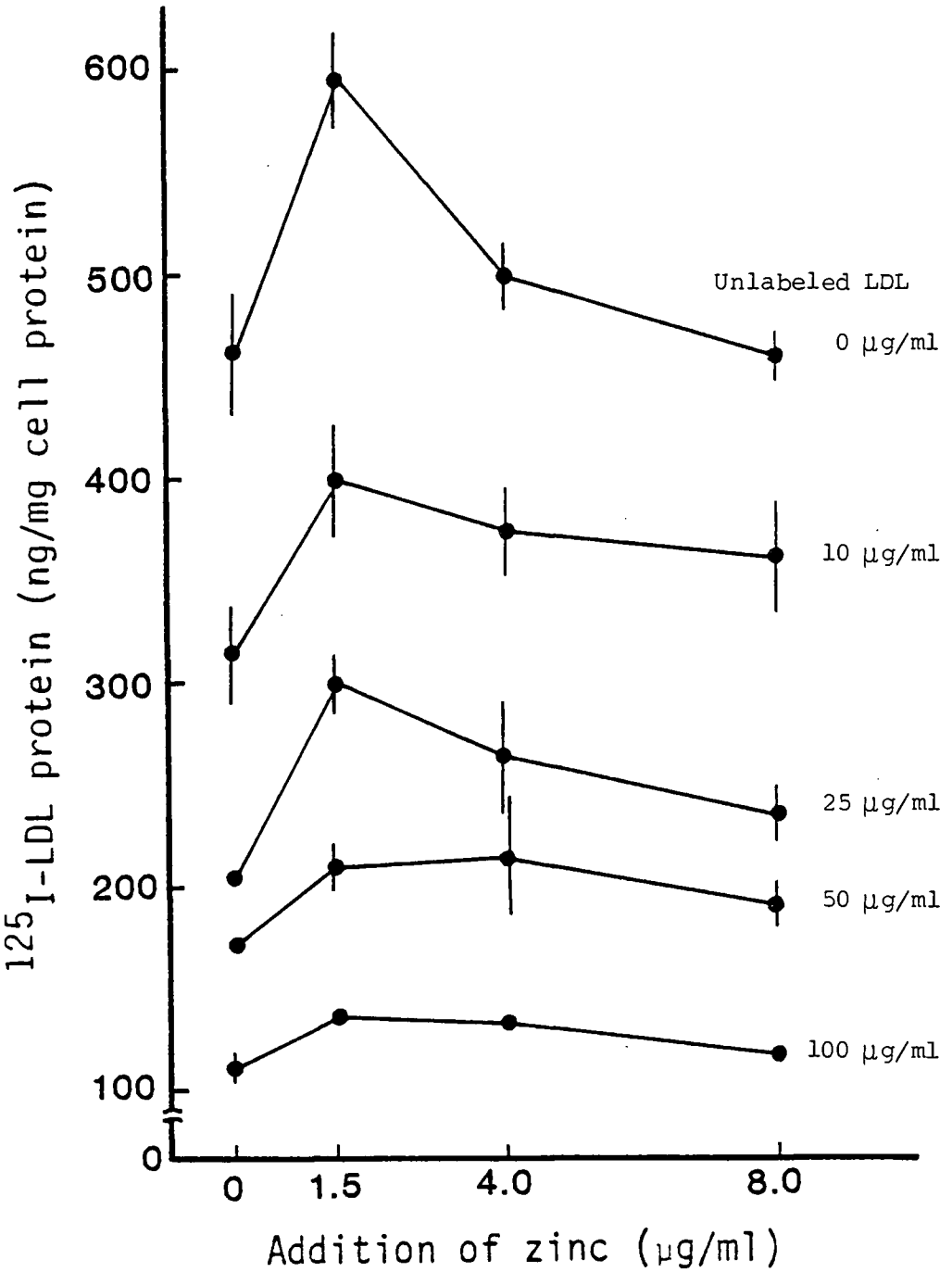

Figure 12. Bound and internalized ^{125}I-LDL to normal human fibroblasts grown in various zinc concentrations. Each point represents the mean of the three culture dishes and standard deviation. All dishes contained ^{125}I-LDL (5 µg/ml) in addition to the indicated amount of unlabeled LDL.

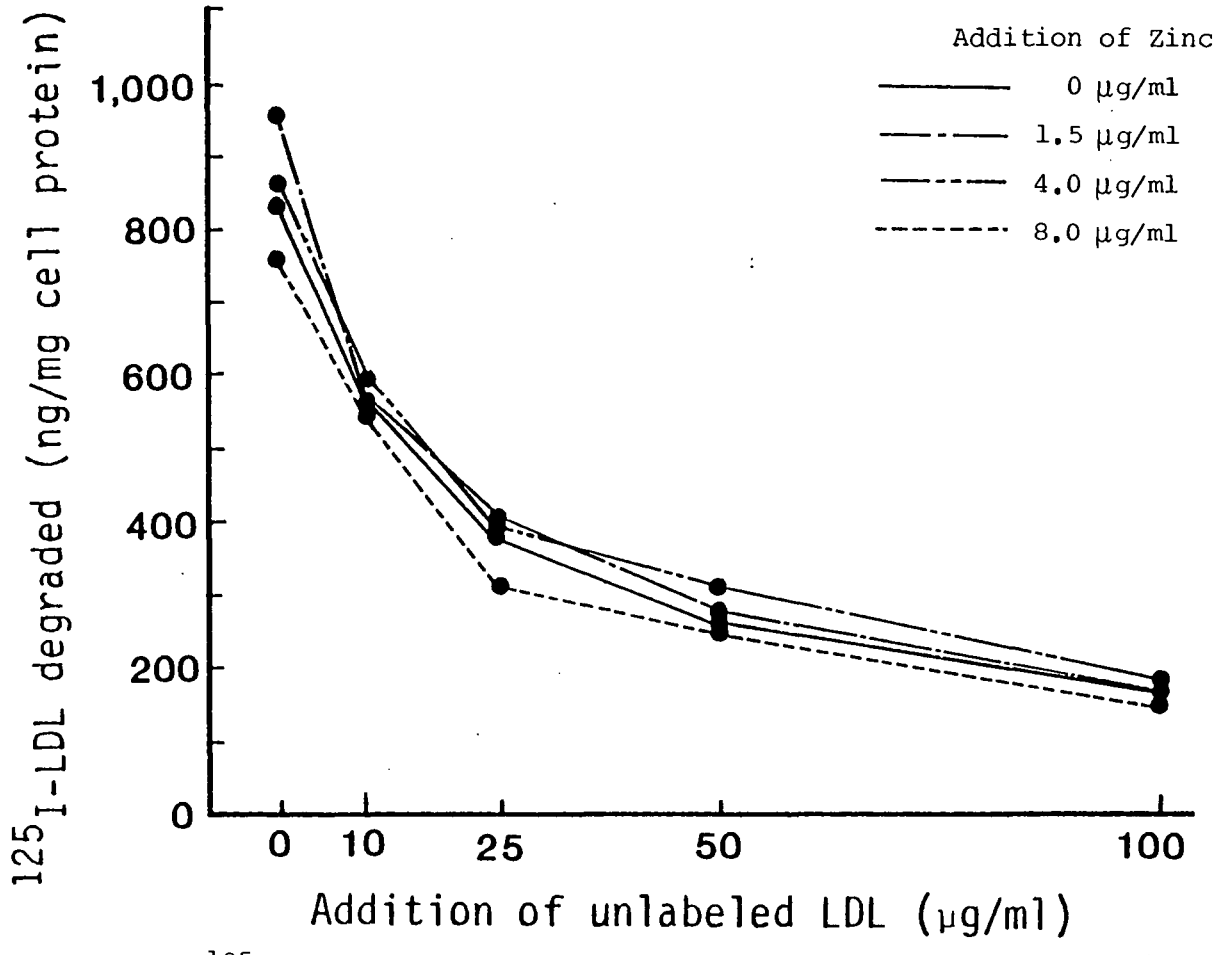

Figure 13. Degradation of ^{125}I-LDL by normal human fibroblasts grown in various zinc concentrations. Each point represents the means of the three culture dishes. All dishes contained ^{125}I-LDL (5 µg/ml) in addition to the indicated amount of unlabeled LDL.

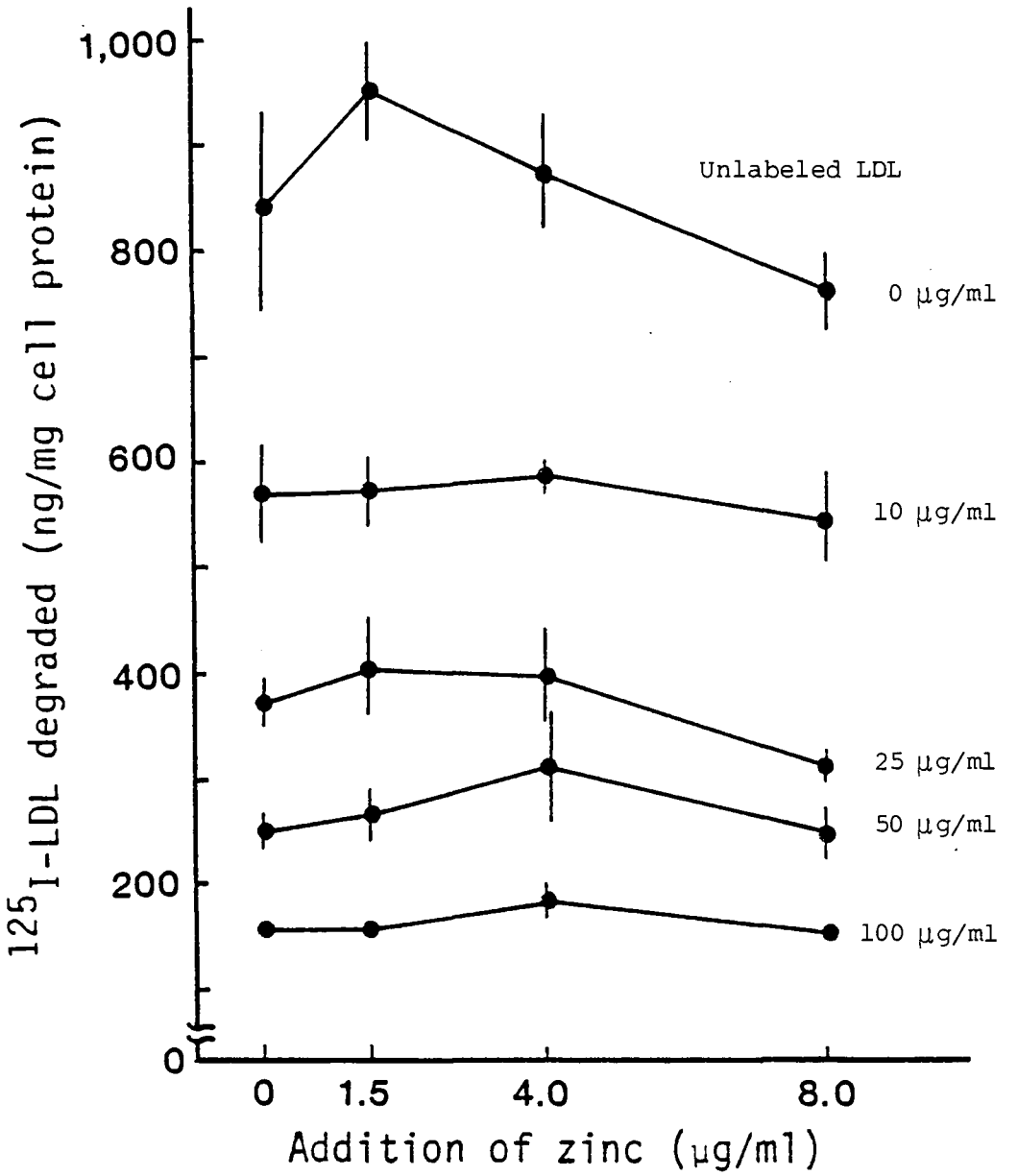

Figure 14. Degradation of ^{125}I-LDL by normal human fibroblasts grown in various zinc concentrations. Each point represents the mean of the three culture dishes. All dishes contained ^{125}I-LDL (5 µg/ml) in addition to the indicated amount of unlabeled LDL.

Table 9. Means of bound and internalized ^{125}I-LDL to normal human fibroblasts (^{125}I-LDL protein ng/mg cell protein)

Addition of Zinc (μg/ml)	Addition of Unlabeled LDL (μg/ml)				
	0	10	25	50	100
0	457 ± 31	311 ± 24	202 ± 3	171 ± 1	111 ± 6
1.5	598 ± 23	398 ± 27	300 ± 16	208 ± 12	135 ± 2
4.0	499 ± 16	375 ± 21	261 ± 29	213 ± 30	190 ± 11
8.0	460 ± 13	361 ± 26	235 ± 16	190 ± 11	118 ± 4

Each dish contained 5 μg/ml ^{125}I-LDL in medium in addition to the indicated amount of unlabeled LDL.

Table 10. Means of degraded ^{125}I-LDL by normal human fibroblasts (LDL protein ng/mg cell protein).

Addition of Zinc (μg/ml)	Addition of Unlabeled LDL (μg/ml)				
	0	10	25	50	100
0	837 ± 97	568 ± 49	373 ± 23	252 ± 18	167 ± 8
1.5	955 ± 48	568 ± 34	401 ± 42	263 ± 23	167 ± 3
4.0	869 ± 49	588 ± 15	398 ± 38	310 ± 56	180 ± 14
8.0	756 ± 40	543 ± 44	312 ± 1	247 ± 24	150 ± 8

Each dish contained 5 μg/ml ^{125}I-LDL in medium in addition to the indicated amount of unlabeled LDL.

Table 11. Means of cholesterol content in cells after LDL binding (cholesterol μg/mg cell protein).

Addition of Zinc (μg/ml)	Addition of Unlabeled LDL(μg/ml) 0	10	25	50	100
0	25.00± 7.02	24.40± 2.02	27.15± 1.32	33.57± 1.71	36.29± 3.59
1.5	30.36± 3.17	24.98± 2.71	36.25± 2.02	33.32± 2.65	35.00± 5.81
4.0	26.65± 1.62	31.05± 3.09	30.85± 5.49	36.58± 11.22	31.90± 5.18
8.0	28.17± 2.04	27.94± 0.80	28.61± 1.54	31.68± 3.37	30.29± 2.41

Cholesterol was assayed by a gas chromatograph(Hewlett Packard, model 5710A)

DISCUSSION

Since the discovery of low density lipoprotein (LDL) receptors in 1973, plasma cholesterol metabolism has been extensively studied in cultured mammalian cells. As a result, the regulatory mechanism of plasma cholesterol levels as mediated by receptor endocytosis of LDL was revealed. However, there is very limited information regarding the association of lipoprotein transport and other nutrients, including minerals. Zinc, an essential element for plants, animals, and humans, has been known to participate in a wide variety of metabolic processess including protein, nucleic acid, and carbohydrate metabolism, but again there is limited information concerning the interaction of zinc and lipids. In the present study, the effect of zinc on cell proliferation and receptor mediated LDL metabolism in human fibroblast cells was investigated.

The standard medium composed of DMEM, 5% (v/v) FCS and 1% (v/v) of penicillin-streptomycin was used for all the cell growth studies and the pre-incubation period of cells for the LDL binding study. The regular medium used for cell growth studies I and II contained 0.21 μg/ml of zinc. When cells were incubated in the medium supplemented with less than 4 or 6 μg/ml of zinc, the number of cells and protein content in the cells did not differ from the controls, indicating no stimulatory effect of zinc on cell proliferation. However, additional increases of zinc to the medium above 6 μg/ml lowered the number of cells in the dishes and cellular protein content.

Falchuk et al. (1975) reported that suppression of cell proliferation of Euglena Gracilis at low concentration of zinc in the medium 1×10^{-7}M (0.0065 μg/ml) was observed and 1×10^{-5}M (0.65 μg/ml) of zinc added to the medium was sufficient to support cell growth. Although their experimental design was quite different from the design of this study, the findings seem to suggest that it is possible to obtain acceleratory effects by addition of zinc to medium if the zinc content of the regular medium initially is greatly reduced.

Although zinc is omitted from the components of commercial DMEM, a small amount of zinc (0.08 μg/ml) was present because zinc associated with other components in the medium. The FCS used in the study contained substantial amounts of zinc, ranging from 2.67 to 4.10 μg/ml. More than half of the amount of zinc in the regular medium which consists of DMEM and 5% (v/v) was derived from zinc in the FCS.

A chelation technique for trace metals previously described by Messer et al. (1982) was chosen to remove substantial amounts of zinc in FCS and small but measurable amounts of zinc in DMEM. The FCS and DMEM were individually treated with Agarose beads firmly coupled with iminodiacetate, a chelator which binds divalent cations. Details of the method have been described in the section in materials and methods. The advantage of the present method is that zinc and copper can be selectively extracted from both medium and serum. Therefore, if a single trace metal removal is desirable, either zinc or copper can be added to reconstitute to the original concentration. In addition, by using this method, cells are not exposed directly to the chelator, as happens when other chelating agents such as EDTA are added to the medium followed by the addition of a single trace metal.

In the present study, about 90% of the zinc in FCS and 50 to 60% of the copper were removed by the method, but unexpectedly substantial amounts of calcium and manganese were also removed. This suggests that selective extraction of zinc and copper from FCS was not attained. On the other hand, selective extraction of trace metals in DMEM appeared to be successful because 63% of the zinc was removed and 17% of the copper was removed while other divalent cation concentrations remained the same. The final culture medium consisting of DMEM and 5% (v/v) FCS contained very low levels of zinc while other divalent cations were kept the same. This was possible because most of the zinc content in FCS and DMEM was removed by the treatment while the calcium and manganese contents in DMEM (which is 95% of the culture medium) was not changed by the extraction.

Serum from healthy humans contains 0.84 to 1.20 μg/ml of zinc ions(Lyenger et al.,1978). Approximately 66% of serum zinc is loosely bound to albumin and 32% is tightly bound to α_2-globulin (Subcommitte, 1979). Zinc ions in the serum also bind other protein ligands such as ceruloplasmin and transferrin(Prasad, 1979). The protein content in the FCS(control lot number 27K5501) was slightly reduced by the metal extraction from 5.06 g/dl to 4.00 g/dl, while the zinc concentration was greatly reduced to 20% of the original FCS concentration. This result indicate that the affinity of the protein ligands for zinc ions in the FCS may be lower than that of the binding site of the chelating agent, iminodiacetate.

The cell growth study(Study III) with zinc supplementation of the zinc extracted media showed the same trend obtained by the cell growth study using the intact media. There was no stimulatory effect of low levels of zinc addition, indicating that the 0.045 μg/ml zinc present in the standard growth medium was sufficient to support cell growth. A toxic effect of zinc on cell proliferation was observed at concentrations above 6 μg/ml of zinc addition. In this study the toxic effects of zinc on the numbers of cells as well as the protein content in cells appeared to be moderate compared to the results obtained from studies I and II which used unextracted media. This observation suggests that there is a moderately toxic effect at high zinc levels on cell proliferation. However, cell protein content with each zinc level in this study was much higher than that obtained in studies I and II. The experimental design of study III, and that of studies I and II may explain the difference in the results obtained. In study III, cells were incubated two days longer than in studies I and II, and the growth medium was changed more frequently. Furthermore, the cell suspensions were sonicated before the protein analysis, whereas in studies I and II cell suspensions were frozen and thawed twice. Sonication seemed to produce better preparations for analysis of cellular protein. Although the magnitude of the overall data in study III is different from the data obtained in studies I and II, the results of study III can be interpreted in the same way: addition of more than 6 μg/ml zinc to the

growth medium has a toxic effect on protein synthesis and cell proliferation.

The role of zinc has been studied in cells from different animals and different types of cells. The deficient or stimulatory effects of zinc ions on cell growth were reported by several investigators. Zinc concentrations of from 1.5 to 4.5 x 10^{-4}M (9 to 29 μg/ml) of zinc in the medium had a stimulatory effect on mitosis of lymphocytes (Chvapil, 1976). An addition of 10 μM of $ZnCl_2$ (0.65 μg/ml) enhanced mitosis of hamster lymph node cells (Hart, 1978). Zinc concentrations of from 0.1 to 0.2 mM (6.5 μg/ml to 13.1 μg/ml) stimulated DNA synthesis in lymphocytes (Chvapil, 1976a).

In spite of the stimulatory effect of high zinc concentration on cell growth as mentioned above, one study indicates the toxic effect of zinc at low concentrations. Waters et al. (1971) reported that the addition of 10^{-4}M, 10^{-6}M, and 10^{-8}M (6.5 μg/ml, 0.65 μg/ml, and 0.65 ng/ml) of zinc to the culture medium suppressed collagen biosynthesis and proliferation of human skin fibroblasts. Epstein (1982) observed a toxic effect of zinc on human fibroblasts at a rather high concentration of zinc, 0.2 mM (13 μg/ml).

Due to the limited findings in the literature and inconsistency of results regarding the interaction between zinc levels in culture media and cell growth, it is difficult to define deficient, optimal, or toxic levels of zinc in the medium for growth of various cells. This inconsistency is probably because of differences in cell types, medium composition, incubation periods, and other variables.

The following information may help to clarify why zinc addition of greater than 6 μg/ml to the medium suppressed cell growth in this study. Zinc at a low concentration of 2 μM (0.13 μg/ml) inhibits the electron transport system between cytochrome b and c_1 in the mitochondria respiratory chain (Chvapil, 1976). Zinc at 0.5 mM (33 μg/ml) inhibits ATPase activity (Chvapil, 1976). Because ATPase is an important enzyme for energy dependent activity in many cells, inhibition of ATPase activity by zinc along with failure in the electron transport system may result in a change in the integrity of the

mitochondrial membranes, which may in turn cause suppression of cell growth.

Although the LDL binding and degradation study was designed to investigate how zinc status changes the ability of ^{125}I-LDL to compete with unlabeled LDL for binding and degradation, the results of the total LDL bound to the cells and degraded by the cells show an effect of zinc status on the binding activity of LDL to the cell surface receptor. When competition of LDL and ^{125}I-LDL was altered by zinc treatment of cells, the binding of LDL and degradation were changed in the same manner. Maximal binding of ^{125}I-LDL to the cells and degradation of ^{125}I-LDL by the cells were observed in cells preincubated in the medium with 1.5 μg/ml of zinc. Further increases in zinc addition to the medium during the incubation period prior to the LDL binding caused a decreased amount of ^{125}I-LDL to be bound to the cells and degraded by the cells, indicating an inhibitory effect of high levels of zinc associated with cells on the binding sites of LDL.

There was no zinc effect on total cholesterol levels in cells after the LDL binding. There was a trend to have higher cholesterol content in cells incubated in higher levels of LDL in each zinc treated group. The changes in cellular cholesterol content by the incubation of cells with LDL has been reported by Brown et al.(1975). When human fibroblasts were incubated in medium devoid of lipoproteins for 24 hours, the cellular content of esterified cholesterol was between 1 to 2 μg/mg cell protein and that of free cholesterol was approximately 20 μg/mg cell protein. When cells were incubated in the presence of LDL (375 μg of LDL-cholesterol) for 24 hours, cellular content of esterified cholesterol increased by 7-fold while cellular free cholesterol level increased only by 1.6-fold. Therefore, the slight increase in cellular cholesterol content observed in this study is probably due to an increased amount of incoming cholesterol derived from LDL in the medium.

The results obtained from the studies of cell growth and LDL binding can be summarized as follows. Addition of 1.5 μg/ml zinc to the medium was within the optimal range of zinc concentration for the growth of human fibroblast cells. When cells were grown in the medium with this zinc level, binding of LDL was maximized at low concentrations of LDL. However, high levels of zinc in the growth medium caused toxicity for the growth of cells and hindered normal binding of LDL to the cells. These findings prompt the following discussion.

Bound and internalized zinc ions which exist in the cells prior to the LDL binding may interact with some enzymes and cellular organelles and affect their function which may be needed for receptor mediated endocytosis of LDL. The ATPase activity and electron transport system in mitochondria might be affected by high levels of zinc during the pre-incubation period as mentioned earlier. This might cause some change in the mitochondria and perhaps in other cellular organelle functions. Thus zinc may influence binding and degradation of LDL in fibroblast cells. Brown and Goldstein (1979) described the important role of some cellular organelles in the regulation of cholesterol metabolism of fibroblasts. After LDL receptors are synthesized in the endoplasmic reticulum, they move to the coated region of the plasma membrane. When LDL binds to the LDL receptors, the receptors are internalized as the coated pits invaginate to form coated endocytic vesicles. These vesicles rapidly fuse with lysosomes. There, the LDL protein is hydrolyzed to amino acids, and the cholesteryl esters are hydrolyzed by a lysosomal acid lipase. The cholesterol derived from internalized LDL acts as the primary agent to control the feedback system to stabilize the cellular cholesterol concentration. Three regulatory reactions are described by the investigators. First, the incoming cholesterol suppresses the microsomal enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), the rate-controlling enzyme in cholesterol biosynthesis, so that cholesterol synthesis in the cell is turned off. Second, the incoming cholesterol activated a microsomal enzyme, acyl-CoA: cholesterol acyltransferase (ACAT), an intracellular cholesterol

esterifying enzyme. Therefore, excess cholesterol can be reesterified and stored as a cellular component. Finally, the incoming cholesterol turns off the synthesis of the LDL receptors, preventing uptake of LDL to the cells. In view of the importance of cellular organnelles for participation in the regulation of cellular cholesterol content, zinc, if it alters the function of cellular organelles, could influence LDL binding and degradation.

Several investigators mention the protective effect of zinc on cell membrane lipid peroxidation, either by interfering with the initiation step (Prasad, 1979) or protection from toxic superoxide anion by incorporation into a zinc metalloenzyme, superoxide dismutase (Fee and Teitelbaum, 1972). If the series of steps in the receptor mediated endocytosis mentioned above requires a healthy cell membrane, then zinc probably plays an important role in controlling the LDL binding activity of cells through its influence on cell membrane condition.

Binding of apo B protein (main protein in LDL) is inhibited by acetylation of lysine residue (Brown et al., 1981) or by modification of arginine residues by cyclohexanedione (Mahley et al., 1977). These findings suggest that lysine and arginine residues of apo B protein in the human LDL are recognition sites for the LDL receptors on the surface of human fibroblasts. Goldstein and Brown (1974) and Basu et al. (1978) studied the interaction of calcium ions and LDL in the medium and its influence on LDL receptor on the cells. They found that at low levels of LDL in the medium, the addition of calcium enhanced both binding and degradation of LDL. However, the mechanism of the enhancement, whether calcium interaction with apo B or alteration of conformation of LDL receptors, has not been revealed yet.

In this study, zinc was not added to the LDL binding medium; therefore, neither the interaction of zinc and apo B nor competition of zinc with LDL for binding to the LDL receptors was investigated. Zinc has not been found to interact with amino acids in the serum proteins other than histidine (Subcommittee on Zinc, 1979). However,

if there is an interaction between zinc and lysine or arginine residues of LDL protein, zinc will probably affect the binding of LDL to the receptors by altering charges on the apo B or by other actions. Therefore, if zinc were allowed to bind directly with LDL by adding it to the LDL binding medium, the results might be different from those obtained in the present study.

Summary and Conclusions

There was no stimulatory effect of zinc supplementation of media that had or had not had zinc removed on cell growth and cell protein content. Zinc concentration in the zinc removed media was 0.045 μg/ml and was enough to support normal cell growth. Higher levels of zinc supplementation had toxic effects on cell growth as well as decreased LDL binding to cell receptors. Since normal human serum contains 0.84 to 1.20 μg of zinc per ml (Lyenger et al., 1978), it was not surprising to see no effect of the low level of zinc supplementation on the growth of cells. However, when cells were grown in the media with zinc removed and supplemented with 1.5 μg/ml of exogenous zinc, high affinity binding of LDL to the cell receptors was maximized. These results suggest that zinc may be involved in the binding process of LDL to the receptors. This finding suggests that the low level (1.5 μg/ml) of serum zinc may be important in controlling serum cholesterol levels. The zinc involvement in the LDL binding process can be hypothesized as follows: zinc may change structural stability or function of cellular organelles, including LDL receptors, which are required for receptor mediated endocytosis of LDL; or zinc may regulate the energy transport system, thus controlling whole cellular activity. Further study will be required to evaluate these hypotheses and reveal details in the mechanism of zinc involvement in the LDL binding system.

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