AN ABSTRACT OF THE THESIS OF

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Selenium (Se) is one of the dietary trace minerals necessary in the diet to maintain health of the animal. Se-deficiency causes muscular dystrophy called "white muscle disease" (WMD) in young lambs and calves. Since the exact role of Se in skeletal muscle cells is still unknown, the mechanism of induction of WMD is still unclear. Most previous studies of Se-deficiency on skeletal muscle has been done *in vivo*, and detailed investigations on biochemical aspects of this syndrome have been limited.

The first goal of this study was to develop skeletal muscle cell culture study model using serum-free (SF) media which supported both differentiation and myotube viability. Serum-free media consisted of DMEM supplemented with insulin (10^{-7} M), transferrin (5mg/l), dexamethasone (10^{-7} M), linoleic acid (1mg/l), and fetuin (300mg/l) . Omittion of each of the components from DMEM media caused 96, 50, 40, 22, and 6 % reduction in the differentiation rate assessed by creatine kinase activity, respectively. Thus, this media was used to create a low Se extracellular environment for L8 rat skeletal muscle cells. Effects of Se concentration (0 versus 2.5×10^{-7} M; added as Na-selenite) on cellular Se status were investigated by assaying cellular Se content, glutathione peroxidase (GSH-Px)

activity, and selenoprotein W expression. Removal of Se from culture media reduced both muscle cell Se concentration and GSH-Px activities within 24 hours of the treatment. Selenoprotein W expression was detected 24 hours after Se supplementation and was not detected without Se. Hence, removal of Se from culture medium provides a model for studying the biological function of Se in vitro.

The second goal of the study was to evaluate the effects of Se on the rate of differentiation, protein degradation, and cell detachment (death) in cultured L8 muscle cells. To assay the effects of Se on differentiation, we examined effects of Se (0 versus 2.5×10^{-7} M) on muscle cell CK activity for period of 96 hours. This and following experiments were repeated at least three times. Following removal of Se from culture medium for 96 hours, CK activity was reduced ($P<0.05$) by approximately 15 %. Hence, Se is required for optimal muscle cell differentiation. To evaluate effects of Se on protein degradation in myotubes, we examined effects of three Se concentrations (0, 2.5×10^{-7}, 2.5×10^{-6} M) on degradation of proteins in cultured myotubes. Protein degradation was assessed as release of ^{3}H-labelled free amino acids secreted into the media. Se supplement had no affect ($P>0.05$) on total protein degradation. Hence, Se does not significantly influence protein turnover in L8 muscle cells. However, we found a significant increase in loss of cells by Se-deficiency, which is indicated by cell detachment. We propose this may be the basis for muscle cell death during Se-deficiency syndrome.

We also assessed the ability of vitamin E to compensate for Se-deficiency. Confluent myoblasts were treated with SF media with one of four different levels of vitamin E (0, 10, 35, 100 μM) in the absence and presence of Se (0 and 2.5×10^{-7} M). Cells were incubated for 96 hours and differentiation was assessed by CK activity assay.

Vitamin E at a higher dose (100 μM) was partially effective in preventing the decrease of differentiation caused by Se-deficiency ($p<0.05$). Vitamin E treatment at any other dosage were not effective in preventing cell detachment caused by Se-deficiency.

These results indicates that Se is certainly necessary for development and maintenance of skeletal muscle cells. Also, the serum-free cell culture system allows further investigation of biochemical aspects of WMD, and will provide a general basis for understanding of muscular dystrophic diseases.

Effects of Selenium on Differentiation and Degeneration of Cultured L8 Rat Skeletal Muscle Cells

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Dedication

This thesis is dedicated to my wife, Shiomi, for her endless love and patience, and also to our children, Akeno Andrea and Alexander Ryo, for providing me with encouragement by their endless smiles.

Effects of Selenium on Differentiation and Degeneration of Cultured L8 Rat Skeletal Muscle Cells

Chapter 1. Introduction and Literature Review

Introduction

Although the element selenium was discovered in 1817, its biological importance was not recognized until more than a century later. Interest was focused on its potent toxicity against cattle and other livestock in the 1930's. Experiments by Franke in 1934 identified selenium toxicity as a cause of "alkali disease" in livestock (Franke, 1934). The other common Se toxicity syndrome is called "blind staggers". This disease is found in animals consuming smaller amounts of plants which contain a high level of Se over a short time (Shamberger, 1983).

It was not until the 1950's that selenium was identified as an important nutrient (Schwarz, 1957). Soon after that finding, animal nutritionists began to recognize that some of the livestock diseases were the result of Se deficiency. Various organs in different animals were shown to be affected by Se deficiency (Shamberger, 1983). Many selenium deficiency diseases are related to degeneration or necrosis of certain tissues. In lambs and calves, muscular dystrophy of skeletal muscle has been the most crucial problem associated with Se deficient animals (Muth, 1963). This condition, commonly called white muscle disease (WMD), is characterized by a white streak of necrotic muscle tissue found mainly in hind leg of the affected animal. Since limb muscles are one of the most valuable portions of these livestock, as a meat product and as means of survival of

that animal, WMD has been studied extensively by many animal nutritionists and agricultural chemists.

The exact mechanism of most Se deficient symptoms in animals is not clearly understood, including its role in WMD. However, Se deficiency diseases has been shown to be treatable by supplementing Vitamin E to the animal (NRC, 1983). Vitamin E is an antioxidant and protects cells from harmful peroxides. Later, selenium was also identified as an antioxidant, acting as an essential component in the family of enzymes called glutathione peroxidase (GSH-Px) in eukaryotic cells (Stadtman, 1990). Although, Se as in GSH-Px contributes to maintenance of cell integrity, not all Se-deficient pathophysiological conditions are explainable by the lack of GSH-Px.

In addition to GSH-Px, three other proteins which contain Se as an essential component (selenoprotein) have been discovered. Those are: selenoprotein P, selenoprotein W, and thyroid hormone deiodinase. Of these three selenoproteins, the functions of the first two have not been elucidated. It is essential to find the roles of these selenoproteins to clarify the mechanisms of Se deficiency-induced pathological conditions. It is also possible that there may be other Se containing proteins yet to be discovered (Behne et al.,1995) .

Although WMD has been studied for over 40 years, *in vivo* models have not permitted identification of the biochemical basis for this disease. Skeletal muscle cell culture allow identification of important information related to the biochemical, and molecular aspects of Se-deficiency syndromes. Since all cells are generated from one batch of original cells, cell culture model can provide consistent data over repeated

experiments. Also, cell culture system allows generating Se-deficient muscle cells within two weeks, where it sometime takes years to obtain WMD livestock animals.

Literature Review

Selenium- Overview

Selenium, molecular weight 78.96, is classified in group VI-A in the periodic table of elements. Sulfur is also located in this group and these elements share similar chemical and biological properties. Selenium is found in virtually all naturally-occurring materials on the earth, of which the greatest abundance can be found in igneous rocks. Minerals are one of the richest sources of selenium. Sedimentary rocks also contain Se in high concentrations (NRC, 1983). Although soils normally contain lower Se than rocks, soil Se concentration is far more important to biological systems. Since plants, which are at the bottom of food chain, take up selenium from the soil, the level of dietary intake of grazing livestock and humans usually corresponds to the level of soil Se concentration where main food products are supplied locally (Ullrey, 1981). Soil types are usually classified into three categories based on the toxicity level of Se contained in the soil. The first type is called "toxic seleniferous soils". This type of soil contains free calcium carbonate and is usually alkaline which is ideal for forming a soluble form of selenium, thus leading to growth of a high selenium plant (Rosenfeld et al., 1964). Rosenfeld lists that, in the U.S., toxic selenoferous soils are found in most of western states except Pacific coast states. She also mentions that the indicator plants, which literally accumulate and indicate the high selenium concentration of the soil, can be found in Wyoming and South Dakota (Rosenfeld et al., 1964). The average Se concentration of 500 soil samples from seleniferous area in U.S. was 4.5 ppm with maximum of 80 ppm (Trelease, 1945).

The second type of soil is called "non-toxic seleniferous soil". This type of soil is only reported in Hawaii and Puerto Rico with Se levels of 6 to 15 ppm and 1 to 10 ppm, respectively. Although this concentration is relatively high, water soluble selenium levels are low and soil is acidic, which makes selenium unavailable to plants (reviewed in Ullrey, 1981).

The third type, "low selenium soils", covers mainly coastal areas of the U.S., of which, very low selenium soil can be found in the Pacific Northwest and South Atlantic seaboard. Most of low-selenium soils contain less than 0.5 ppm of Se (NRC, 1983). In the other countries, such as New Zealand, large parts of soils contain between 0.1 and 2 ppm Se, and it is likely that soils of Finland and certain regions of China contain low levels of selenium (summarized in NRC, 1983). Obviously, plants grown in these area will be low in Se, causing low dietary selenium intake by animals and humans of that region or country.

Kubota et al. (1967) has mapped the selenium levels of crops (mainly alfalfa) in the U.S. The map clearly shows that Se concentration of the samples obtained from low selenium soils are very low (less than 0.05 ppm) compared to the samples obtained from Se adequate regions (0.23 ppm, Kubota et al., 1967). He also pointed out that the incidence of WMD can be found primarily in the Pacific Northwest region. However, there is a very low incidence of WMD in Atlantic seaboard. He suspects that the high level of vitamin E found in this region is compensating for Se deficiency.

Low selenium content in soil also causes Se deficiency problems in human. Human dietary selenium intake has been found to be low in New Zealand and Finland, and

extremely low in certain regions of China where a human selenium deficiency disease (Keshan disease) has been reported (Levander, 1987). In the U.S., certain important food crops, such as wheat, are primarily grown in selenium adequate regions, and human dietary selenium intake is therefore sufficient (Ullery, 1981).

Biological availability of selenium in the diet is largely dependent on its chemical form in the feedstuff (Mertz, 1986). The form of selenium is the main factor affecting the absorption and utilization of selenium in animals or humans. To date, more than ten naturally-occurring low-molecular weight selenium compounds have been identified. Shrift (1969) listed Se compounds which can be found in higher plants: Selenocystine, selenocysteine, Se-methylselenocysteine, selenohomocystine, selenomethionine, Se-methylselenomethionine, selenomethionine selenoxide, selenocystathioneine, and dimethyl diselenide. However, of these selenium compounds, selenocystine, selenocysteine, selenomethionine, and Se-methylselenomethionine are more abundant in forages and seeds commonly consumed by animals (Petersone et al., 1962; Shrift, 1969). Although organic selenium appears to be the major source of forage and seed Se, inorganic Se, such as selenate and selenite, are also used as a dietary supplements to animal feed (Podoll et al., 1992). Numerous synthetic selenium compounds have been developed (Shamberger, 1983). However, these synthetic compounds are mainly used as potential chemotherapeutic agents and not for dietary purposes.

Several studies have shown the metabolic differences of organic versus inorganic forms of selenium (Butler et al., 1989; Deagen et al., 1987; Rousseaux et al., 1993; Swanson, 1987; Vendeland et al., 1992). For comparison, they normally use

selenomethionine (and to a lesser extent, selenocystine) as an organic form of selenium and selenite as an inorganic selenium. Although sodium selenate has been shown to be absorbed and metabolized slightly faster than sodium selenite in various animals, the overall effect was very similar for practical feeding (Vendeland et al., 1992) Therefore, sodium selenite will continue to be the primary inorganic selenium supplement used in the feed (Podoll et al., 1992). To summarize, selenium is concentrated into various tissues fastest and retained longer when animals are fed selenomethionine, compared to selenite. However, glutathione peroxidase activity, so far the only direct way to assay utilization of Se in tissues, is not different between animals fed different forms of selenium (Deagen et al., 1987; Podoll et al., 1992; Shan et al., 1994; Butler et al., 1989). Therefore, it has been suggested that more Se is incorporated into non-GSH-Px proteins from selenomethionine. Selenocysteine, although it is an organic selenium, has been shown to have similar (Deagen et al., 1986, Osman et al., 1976) or even less (Sunde et al., 1980) biological potency than inorganic selenite rather than that of selenomethionine. Therefore, it's been postulated that selenomethionine and selenite have different metabolic pathways and selenocysteine may follow the pathway similar to selenite (Deagen et al., 1987).

Selenium Deficiency

The essentiality of selenium in animals was first discovered by Schwarz and Foltz (1957). They showed that selenium can protect rat liver from necrotic degeneration. Since then, Se deficiency has been found to cause pathological conditions in most livestock animals including cattle, sheep, swine, horses, turkeys and chickens. Among

these animals, young animals are the most susceptible to Se-deficiency (NRC, 1983). A wide variety of laboratory mammals, from mouse to monkey, also have been tested for responsiveness to Se deficiency.

Signs and severity of Se deficiency vary between species. In rats, as well as in the mouse, liver and kidney necrosis is a major characteristic of a Se deficient diet (Schwarz and Foltz, 1957; De Witt and Schwarz, 1957). In young chickens, turkeys and other domestic avian species, Se deficiency is characterized by fluid accumulation under the skin of the abdomen and breast, and is called exudative diathesis (Dam and Glavind, 1958). Pancreatic degradation, muscular dystrophy, and legions in gizzard have also been reported in these birds (NRC, 1983).

Among the signs observed in Se deficient pigs, a cardiomyopathy called "mullberry heart disease" drew most attention (Piper et al., 1975). This condition is characterized by streaks of necrosis and patches of congestion and hemorrhage, causing weakening of the heart muscle. Rapidly growing young animals, can die suddenly due to heart failure (Shamberger, 1983). This cardiomyopathy is similar to Keshan disease found in Se deficient humans (reviewed by Levander, 1987).

Se deficiency in cattle and sheep is associated with myopathy of both cardiac and skeletal muscles. This disease is generally called nutritional muscular dystrophy for all animals. However, because its clinical symptoms are found in skeletal muscle of affected cattle and sheep, it is also called white muscle disease (Muth, 1963). When a young animal (usually pre-weaning) develops this condition, without proper treatment, the animal may eventually die from either sudden heart failure, or starvation due to inability to stand

to be nursed. The effects of Se deficiency on skeletal muscle will be discussed in a later section.

In addition to the characteristics of Se deficiency syndromes listed here for different species, growth, reproduction, and other measures of animal production are generally impaired (NRC, 1983).

White Muscle Disease

The relationship between white muscle disease (WMD) and selenium deficiency was clarified during the 1960's (Muth, 1963). Since then, the histology of WMD was well documented by use of laboratory and livestock animals fed Se-deficient diets. Although the natural incidence of white muscle disease has been reported in many countries, prior to 1965, it was more commonly found in: Northwestern U.S.A. (Muth, 1963), New Zealand (Hartley, 1963), and some areas of Australia (Walker, 1961). This syndrome can occur in various species but is mainly found in young, rapidly growing lambs and calves. More detailed studies have been completed in lambs than calves (Hungerford, 1996).

Clinical signs of WMD are described by Maas (1996):

> ... NMD (WMD) ... characterized by muscular weakness or stiffness... Most affected animals are able to remain standing only for short periods... Commonly affected muscle groups may include the gastrocnemius, semitendinosus, semimembranosus, and biceps femoris and muscles of the lumbar, gluteal, and neck regions.

Maas (1996) also described histological description of WMD from necropsy findings:

> Skeletal muscle degeneration is characterized by pale discoloration and a dry appearance of affected muscle white streaks in muscle bundles, calcification, and intramuscular edema... Affected muscle bundles are often adjacent to apparently normal or minimally affected muscle.

He also mentioned that the same type of white streaks can be found in heart muscle of affected animals. This myocardial damage may cause heart failure and acute death of the animal.

Etiology of WMD

Major damage to muscle tissue by selenium deficiency is caused by loss of cellular integrity. Deposition of calcium in afflicted muscle tissue is one of the major characteristics of this disease (Bonucci and Sadun, 1973). In muscle cells, among other functions, a major responsibility of Ca^{2+} is initiation of muscle contraction (Ruegg, 1986). During the relaxed state, cytoplasmic Ca^{2+} concentration of muscle is 10^{-7} M, and the level raises to 10^{-5} M during muscle contraction. Sarcoplasmic reticulum (SR) which surrounds muscle fibers is the major storage site for Ca^{2+}, and 90 % of the ion during muscle contraction is expelled from the SR into the cytoplasm (McComas, 1996). Therefore, the SR is an important organelle for cellular Ca^{2+} homeostasis and movement of the ion across SR membrane is strictly regulated by the ion channels and ion pumps found on the membrane.

Thus, in Se-deficiency, the excessive accumulation of Ca^{2+} causes continual muscle contraction, or muscle rigor, which is the first clinical sign seen in WMD lambs (Maas, 1996). This is likely due to loss of the ability to sequester calcium inside of the

SR. In fact, inability of the sarcoplasmic reticulum to sequester Ca^{2+} in Se deficient animal was shown in both skeletal muscle and heart muscle (Tripp et al., 1993; Yu-zhen, 1993). In the study of Tripp et al. (1993), WMD lambs were experimentally produced and Ca^{2+} uptake and ATPase activities of fragmented SR were measured. They found that ATPase activities in WMD lamb did not significantly decrease compared to normal lambs, where as Ca^{2+} uptake by fragmented sarcoplasmic reticulum was reduced 10-fold. Therefore, they suggested that Se deficiency is affecting Ca^{2+} binding proteins which are necessary for the ion to be transferred back into SR. In Yu-zhen's study (1993) using rat heart muscle, both Ca^{2+} uptake and ATPase activity were decreased in the animals fed Se-deficient diet, suggesting that a Ca^{2+}-ATPase complex may be affected by Se deficiency.

Yu-Zhen et al. (1993) also found that lipid peroxide content in sarcoplasmic reticulum of Se-deficient heart muscle actually increased. Lipid peroxide production is usually prevented by antioxidative molecules, including glutathione peroxidase (GSH-Px). In the same study, a decrease in GSH-Px was also found in Se deficient rats. Low blood Se concentration usually causes low GSH-Px activity in animals (Caple et al., 1980). A decrease in GSH-Px activity due to the lack of Se in development of muscular atrophy disease has been indicated by other studies (Brown et al., 1986; Yu-zhen et al., 1993). However, not all animals with low GSH-Px activity caused by low dietary Se intake develop symptoms of Se-deficiency myotrophy, suggesting involvement of other factors in the etiology of this disease (Whanger et al., 1977).

In addition to GSH-Px, Tripp et al. (1993) also found involvement of Se (as selenocysteine) in sarcoplasmic reticulum membrane. Thus, they suggest that there maybe

a functional selenoprotein in the membrane. This strongly suggests that Se deficiency also causes loss of selenoproteins other than GSH-Px which are involved in a Ca^{2+} uptake (or possibly ATPase activity) mechanism. Although there is no evidence to support this so far, this SR membrane protein could be one of the selenoproteins which has been discovered and yet its function is unknown.

Effect of Se-deficiency on Protein Degradation

Although, primary mechanisms of SR damage are still unclear, unsequestered Ca^{2+} in the sarcoplasm could cause detrimental affects in muscle cells, leading to actual clinical pathologies of WMD. One of the causes might be uncontrolled activation of calcium-dependent protease, calpain. Although several isoforms of this enzyme have been identified, two of them are more characterized than the others. These are low and high calcium sensitive calpains called μ- and m-calpains, respectively (Emori et al., 1986). Although the exact activation sequence of calpains is still unclear, under normal Ca^{2+} homeostasis, their activation is suggested to be regulated by autolysis and/or by cleaving of m-calpain by μ-calpain, thus increasing their Ca^{2+} sensitivities (Saido et al., 1994; Tompa et al., 1996). Calpains have been found to be important in myofibrillar protein turnover by their involvement in Z-disc degradation (Goll et al., 1989). Therefore, uncontrolled activation of calpain could cause massive breakdown of myofibrils and muscle damage. In fact, calpain has been suggested to be involved in other types of muscular dystrophies (Kumamoto et al., 1995). Therefore, calpains are likely involved in

development of tissue necrosis by breaking down muscle proteins after being activated by excessive amounts of Ca^{2+} in the sarcoplasm.

Effect of Se-deficiency during Differentiation

The study of the etiology of WMD has been mainly focused on the effect of Se-deficiency on myotubes, or fully differentiated skeletal muscle cells (Tripp et al., 1993). Although direct evidence has not been shown, the effect of Se-deficiency during the differentiation period of fetal skeletal muscles could be contributing to the development of WMD after birth. This is supported by the fact that ewes must be fed Se-deficient diets before and throughout gestation and early lactation periods to ensure the development of WMD in their lambs (Hamliri et al., 1993; Young et al., 1961b; Schubert et al., 1961). The effect of Se-deficiency during skeletal muscle differentiation has never been investigated in detail. However, a number of studies have shown the increased expression of a family of selenoproteins (glutathione peroxidase; GSH-Px), during differentiation of non-muscle cell culture (Baker and Baker, 1992; Shen et al., 1994; Vessen et al., 1995), as well as in cultured skeletal muscle cells (Yeh et al., 1997). These indicate the importance of selenium during this period.

Although the exact mechanism is still unclear, since the other antioxidative enzymes are also up-regulated during this period, the response to oxidative stress during cellular differentiation is hypothesized to cause an increase in GSH-Px expression (Barker and Barker, 1992). There is evidence that cultured skeletal muscle increases lipid oxidation during differentiation which may cause oxidative stress on the cell (Sauro and

Strickland, 1987). Therefore, it is most likely that GSH-Px and its isoforms have an important role in protecting skeletal muscle cells during differentiation.

Selenoprotein

So far, the only functional form of Se is found as selenoproteins. Thus, we focused our attention on selenoproteins in this study. To date, five different selenoprotein have been identified and characterized in eukaryotic cells. Each selenoprotein is briefly reviewed here.

1. Glutathione peroxidase family

Cellular glutathione peroxidase (GSH-Px, cellular-)

Cellular glutathione peroxidase (platelet glutathione peroxidase or simply "glutathione peroxidase") was the first selenoprotein isoform to be identified. Coincidently, GSH-Px was discovered by Mills (1957) in the same year that Se was shown to be essential in animals by Schwarz and Foltz (1957). In the early 1970's, GSH-Px was found to be a selenium-containing enzyme (Rotruck et al., 1971). Cellular glutathione peroxidase and other isoforms of this molecule catalyze the reduction of hydrogen or lipid peroxides (ROOH) with reduced glutathione (GSH).

$$2GSH + ROOH \text{ ----> } GSSG + ROH + H_2O \quad \text{(Michiels et al., 1994)}$$

Free radicals cause oxidation of various molecules and cause cellular damages. Thus, this reaction is very important to protect cells from oxygen-derived free radicals.

The molecular weight of GSH-Px varies between 76 and 92 kDa, depending on species. The enzyme is composed of four subunits of approximately 21 kDa and each subunit contains one selenium in the form of a selenocysteine residue (summarized by Stadtman, 1980). GSH-Px cDNA has been cloned and sequenced, and its gene expression and regulation is being studied (Ho and Howard, 1992). The majority of GSH-Px is found in the liver, and 25 % of total body selenium is present in the liver GSH-Px (Behne and Wolters, 1983). However, GSH-Px is also present in muscle cells which undergo rapid oxygen metabolism. In skeletal muscle cells, GSH-Px can be located in the mitochondria and cytosol (Chan and Decker, 1994). In fact, assessment of GSH-Px activity is, so far, the only way to monitor functional Se status of muscle cells (Tripp et al., 1993; Yu-zhen et al., 1993).

The main function of GSH-Px is to protect cells from hydrogen peroxide (H_2O_2). However, the importance of this enzyme is still controversial. Some researchers suggest that GSH-Px is not critically important, since there are other anti-oxidants that which react with H_2O_2 and are present in tissues at higher concentrations than GSH-Px (NRC, 1983). In contrast, Michiels et al. (1994) emphasized the high efficiency of GSH-Px in protecting cells from oxidative damage compared to other anti-oxidative enzymes.

Phospholipid hydroperoxide glutathione peroxidase (PHGPX)

This enzyme was well characterized by Ursini et al. in 1985. It is a monomer of 23 kDa similar to one subunit of cellular GSH-Px, thus containing one selenocysteine (Ursini et al., 1985). One distinct difference from that of cellular GSH-Px is that PHGPX mainly

catalyzes the reductive inactivation of lipid hydroperoxide on membranes (Ursini et al., 1985). Thus, it is hypothesized that PHGPX protects cellular membranes against oxidative damage. Comparison of cDNA sequences showed that there is approximately 40 % homology between PHGPX and one subunit of GSH-Px and there is a distinct PHGPX gene which is different from the cellular GSH-Px gene (Brigelius-Flohe et al., 1994). Further molecular analysis revealed possible tissue-specific transcriptional regulation of PHGPX (Pushpa-Rekha et al., 1995). Two forms of PHGPX (cytosolic and membrane) can be present in the same tissue, and their molecular bases are identical. Thus they are modified post-translationally (Roveri et al., 1994)

PHGPX has been found in many tissues, including muscle cells at lower concentrations. All tissues examined, kidney and liver have the highest activity (Zhang et al., 1989). Roveri et al. (1992) showed that PHGPX apparently has a critical role in spermatogenesis in testis. PHGPX concentration was shown to be under hormonal control, where the other GSH-Px concentrations are dietary-Se level dependent. This is due to the hormonal control of Se uptake by the testis (Behne et al., 1982).

Plasma Glutathione peroxidase (plasma GSH-Px, GSHPx-P)

As its name indicates, plasma glutathione peroxidase is the major GSH-Px isoform found in plasma (Takahashi and Cohen, 1986). Thus, it is sometimes referred to as the extracellular GSH-Px (Avissar et al., 1994). Although the molecular structure is very similar, plasma GSH-Px was found to be distinct from cellular GSH-Px with respect to its antigenicity (Takahashi and Cohen, 1986). Its cDNA sequence was identified in the early 1990's and revealed an approximately 43 to 49 % sequence homology with cellular GSH-Px of various animals (Takahashi et al., 1990), and is very similar to plasma GSH-Px isoforms of various species (Maser, 1994).

Although plasma GSH-Px has been shown to function as a free fatty acid hydroperoxidase, it is very inefficient (Esworthy et al., 1993). Thus, its function is still unclear. The major source of plasma GSH-Px is apparently in the kidney (Yoshimura et al., 1991; Avissar et al., 1994). There is no reference to the presence or activity of plasma GSH-Px in muscle cells so far.

Gastrointestinal glutathione peroxidase (GSHPx-GI)

The last member of Se-dependent glutathione peroxidase family to be characterized is GSHPx-GI by Chu et al. (1993). It is also a tetramer of 22 kDa subunits which is 66 % homologous to cellular GSH-Px. It catalyzes the reduction of various hydroperoxides, including H_2O_2. With the exception of lower level of expression in human liver, GSHPx-GI mRNA was exclusively detected in human colon and rat gastrointestinal tract and nowhere else to date (Chu et al., 1993).

Although all four isoforms of GSH-Px share similar sequence and activity, their genes have been localized in different chromosomes in human (Chu, 1994). Also, as in the case of GSH-Px versus PHGPX, their gene regulation is independent of each other (Lei et al., 1995). Thus, each GSH-Px can have distinct functions and sites of action. For muscle cells, cellular GSH-Px, and most likely PHGPX also, would be the important and functional GSH-Px isoforms.

2. Selenoprotein P

Although the presence of this protein was predicted a few years earlier, Herrman (1977) finally showed that it was distinct from glutathione peroxidase. This protein was characterized by using ^{75}Se and it was found to be incorporated into plasma, thus named selenoprotein P (Burk and Gregory, 1982). This protein was initially found to have molecular weight of 57 kDa (Read et al., 1990); however, the 45 kDa isoform was recently found and both isoforms exist in two to three different forms in plasma (Chittum et al., 1996).

To date, only rat and human selenoprotein P have been extensively studied. A full-length cDNA was obtained from rat liver and the amino acid sequence revealed 10 selenocysteine residues in this protein (Hill et al., 1991). Although selenoprotein P is expressed in many tissues, the signal peptide found on its N-terminal suggests it is an extracellular protein (Burk and Hill, 1994). It is also suggested that selenoprotein P is delivered to the interstitial space of various tissues (Burk and Hill, 1994).

There are two possible roles of selenoprotein P hypothesized. First, because of its unusually high selenium content and presence in plasma, selenoprotein P maybe work as a selenium transport protein (Motsenbocker and Tappel, 1982). Second, like glutathione peroxidase, selenoprotein P may protects cells (especially membranes) from oxidative damage (Burk and Hill, 1994). Both hypotheses have some supporting evidence but also have some contradictions. Presence and influence of selenoprotein P on muscle cells have never been reported. Since it is an extracellular protein, its influence may not be obvious as intracellular proteins such as GSH-Px.

3. Selenoprotein W

Ever since this protein was recently characterized, selenoprotein W was believed to have a role in the onset of white muscle disease. Thus, it was named selenoprotein W (Venderland et al., 1993). Spectroscopic assay revealed four distinct molecular weight forms (two 9.5 kDa and two 9.8 kDa) of selenoprotein W (Beilstein et al., 1996). Its cDNA sequence confirmed the presence of one selenocysteine residue in a single polypeptide chain (Vendeland et al., 1995).

Although the function of selenoprotein W is still unknown, its binding capability to GSH suggests a possible role in redox reactions, with help of unidentified small molecular weight (45 Da) moiety (Beilstein et al., 1996). Selenoprotein W is present mainly in the cytosol, but can be found on the membrane in small amounts. This protein has been found in rat muscle, spleen, testis, brain, and heart, and also in muscle of various animals (Yeh et al., 1995).

Since selenoprotein W was studied mainly in relation to muscle myopathy, functions of this protein in non-muscle tissue have not been investigated. Because it is present in non-muscle tissues, selenoprotein W must have a function in those tissues as well.

4. Iodothyronine deiodinases (Thyroid hormone deiodinases)

Three types of thyroid hormone deiodinases have been found (abbreviated as D1, D2, and D3). Deiodinases are important enzymes for production of active thyroid hormone (T3) by cleaving one of four iodines from prohormone, tetraiodothyronine (T4). Although thyroid hormone has been studied extensively, its activation mechanism is still unclear. However, it has been shown that deiodinases are necessary for thyroxine activation. cDNA sequencing confirmed the presence of selenocysteine in the catalytic site of D1 and D3 (Berry et al., 1991a; Croteau et al., 1995), and D2 also contains selenocysteine and suggested to be present at the catalytic site (Davey, 1995). Amino acid homology between D1 and D3 is more than 70 % (Croteau et al., 1995). Deiodinases are believed to exits as dimers of approximately 27 kDa protein (Larsen and Berry, 1995).

Deiodinases are expressed mostly in liver and kidney, though D3 has been found to be in muscle cells at lower concentration (Croteau et al., 1995). Because localization of D1 in kidney revealed high concentration of GSH present in its proximity, involvement in redox reaction has been hypothesized. However, anti-oxygenation or peroxidase activity is apparently not involved with this enzyme (Larsen and Berry, 1995).

Although D1 concentration is sensitive to dietary-Se, the effect of a decrease in its concentration had little effect on guinea pigs (Cammack, 1995). In white muscle disease, a decrease of deiodinase activity may be occurring in afflicted animals. However, its known function cannot explain the histological damage of muscle cells. Thus, deiodinase apparently does not have a critical role in WMD.

5. Mitochondrial capsule selenoprotein (MCS)

This protein was found in the mitochondrial membrane of spermatids (Calvin et al., 1981). Even though its presence was known for more than 20 years, the evidence of the presence of selenocysteine in MCS using mouse cDNA was not reported until 1992 (Karimpour et al., 1992). Its molecular weight is approximately 20 kDa; however, alternative splicing and/or multiple transcription start sites generate at least two other rare forms of mRNA. The predominant form is shown to contain 3 selenocysteines, where as the other forms apparently do not contain any (Karimpour et al., 1992). Also, recent studies of rat cDNA analysis showed no selenocysteine residues, due to the absence of several nucleotides which are found in mouse cDNA which have different reading frame (Adaham et al., 1996). However, as in case of mouse, rats may be also have alternative transcription to generate selenocysteine encoding mRNA. This is supported by the fact that the original study showing the relationship of selenium and mitochondrial capsule was using rat spermatids (Wallace et al., 1983). Thus, it is likely that some isoforms of rat MCS contain selenocysteine residue.

Although the role of MCS is still unknown, one of the hypotheses is that selenium is forming S-Se (or Se-Se) bridges between mitochondria, allowing mitochondria to form a sheath covering mid-piece of a spermatid (reviewed by Karimpour et al., 1992). However, if this is the only function, MCS would not have any effect on any other tissues, including muscle cells.

6. Other selenium-containing proteins

Recently, another selenoprotein was identified from human lung adenocarcinoma cells (Tamura, 1996). Although the details of this protein are yet to be investigated, it has been found to have thioredoxin activities.

Behne et al. (1995) have found more than 25 selenium-containing proteins based on SDS-PAGE gel of homogenized tissue from ^{75}Se-injected rats. This study confirmed the presence of previously-described selenoproteins in various tissues, and other unidentified proteins. Some proteins may be a precursor of the other, and some may be bound to a binding protein. Also, they pointed out that some selenoproteins were generated by overdoses of selenium replacing sulphur of non-selenoproteins. Regardless of these assumptions, some of these may be true selenoproteins yet to be identified.

Hypothesis and Objectives

Because WMD is now readily prevented by supplementing Se to animal feed, this disease is now rarely seen, thus the research interest has decreased. In fact, there are less than 10 studies primarily on WMD reported during the last five years and the majority of them were outside of the U.S. (Medline and Biological abstracts search). However, the exact mechanism of induction of WMD has never been elucidated. Although it is known to be an essential element, the role of Se in muscle cell is still unknown. Clarification of how selenium deficiency cause WMD may be the key to understand the role of Se and selenoproteins in muscle cells. Selenium itself is still being studied extensively in the fields such as immunology and oncology. Therefore, the identification of function of Se would be very necessary for them.

It is very difficult to elucidate the etiology of WMD during the skeletal muscle development *in vivo*. Cell culture system can effectively provide detailed biochemical data instead. Hence, we used L8 rat skeletal muscle cell culture in this study. However, to develop Se-deficient environment, it was necessary to develop a chemically defined serum-free medium to support differentiation and maintenance of L8 skeletal myotubes. Therefore, we first discuss the process how we developed our SF medium.

Since WMD lambs are developed from the ewes which are fed Se-deficient diet throughout gestation period, various developmental stages of muscle cells could be affected by Se-deficiency. During the differentiation period, muscle cells increase GSH-Px expression, possibly due to an increased oxidation metabolism (Sauro and Strickland, 1987; Yeh et al., 1997). Thus, GSH-Px and other Se-dependent GSH peroxidases may be

important in protecting cells from oxidative damage during this time. Therefore, we hypothesize that if GSH-Px activity is lowered by Se-depletion of myoblasts, then differentiation may be slowed by the presence of excess oxidative molecules. In our experiment, we monitored activity/concentration of two of the selenoproteins (GSH-Px and selenoprotein W) during differentiation to verify the Se-deficiency status of the muscle cells and examined the effects of Se-deficiency on muscle cell differentiation..

Calcium deposited inside sarcoplasm is one of the symptoms associated with WMD (Maas. 1996). This is due to the inability of cells to sequester calcium in sarcoplasmic reticulum (Tripp et al., 1993; Yu-zhen, 1993). Therefore, we hypothesized that if Ca^{2+} accumulates in the sarcoplasm, then the excess of calcium may activate calcium-dependant protease (calpain) which increase muscle protein degradation. This will eventually cause loss of muscle cell integrity, thus leading to the tissue necrosis. To show this, we assessed effects of Se-deficiency on muscle cell protein degradation.

Chapter 2. Development of Serum-Free Conditions for Maintenance of Cultured Muscle Cells

Many studies have been conducted *in vivo* to identify the role of selenium and the functions of selenoproteins in animal tissues. Although use of live animals is essential for biological research, it sometimes requires intensive labor, large amounts of money and long periods of time, especially when livestock are being studied. A cell culture model, referred to as an *in vitro* model throughout this thesis, is an ideal alternative to study biochemical effects of Se deficiency in specific tissues. An *in vitro* model can provide essential information about tissue metabolism at the biochemical and molecular levels with the advantages of time, economy, and handling efficiency, compared to *in vivo* study.

Cell lines of many tissue types are available commercially. Briefly, these cells are usually plated onto a culture plate and fed nutrient media. The nutrient medium varies depending on the cell line; however, it usually consists of two components: basal media and animal serum. All basal media are chemically defined. Animal serum is also characterized, although some plasma components are yet to be identified. Animal serum provides many factors and hormones for cellular metabolism. At the same time, because of the complexity of the serum component, it often interferes with the study of a chemical factor or hormone of interest (Barnes, 1980b), and serum contains selenium as well (Beilstein et al., 1987). Serum Se concentration of the animal could vary by a factor of 10 and greatly depending on the diet of the animal (Beilstein et al., 1987). This is a major disadvantage of using animal serum in cell culture studies of selenium. We usually do not have control over the diet of the animal of serum source. Selenium level could change

significantly if different lots of animal serum were used between experiments. It is ideal to use the same serum for a series of experiments; however, this is sometimes not possible because of the storage and availability of serum. For this reason, use of serum-free (SF) medium was proposed to study the effects of Se deficiency *in vitro* throughout this study.

General application of SF media greatly advanced during the 1980's due to improvement of purification processes for serum proteins (Barnes, 1987). There have been SF medium developed for various cell types which maintain and control growth and differentiation of cells (summarized in Barnes, 1987). Each cell type has different requirements for SF medium composition. However, insulin and transferrin have been found to be essential for virtually all cell types (Barnes and Sato, 1980b).

Selenium has been added to serum free media of various cell types. In fact, a mixture of insulin, transferrin, and selenium is routinely used for serum-free medium for various cell types and is sometimes referred to as ITS mix (Guilbert and Iscove, 1976; Jumarie and Malo, 1991). However, most serum-free studies have focused on the investigation of essentiality of insulin and transferrin, but not selenium (Honegger et al., 1979; Barnes and Sato, 1979; Barnes and Sato, 1980a). Therefore, the role of selenium in many cell culture methods, including muscle cells, is still unclear.

Fewer studies, using serum-free media, have been reported for skeletal muscle cells compared to other cell types (Barnes and Sato, 1980b). Insulin is contained in all serum-free medium for skeletal muscle cells, and transferrin is also contained in many studies except ones for L6 skeletal muscle cells (Dollenmeier et al., 1981; Florini and Ewton, 1981; Florini and Roberts, 1979; Allen et al., 1985). Allen et al. (1985) studied the

essentiality of each component of their serum-free medium in rat skeletal muscle satellite cells. They measured the fusion rate and total number of nuclei to assay the growth and differentiation after omitting each component from the medium. They also assessed the optimal dose of each component for maximum differentiation. However, they did not test selenium in their experiments, though it was supplemented in their serum-free media. Although the serum-free media for L8 rat skeletal muscle cells has never been developed, we have found Dr. Allen's serum-free medium for satellite muscle cell can work well with slight modifications. The medium allowed the cells to undergo differentiation and maintained myotubes.

Thus, in this chapter, we first confirmed the Se-depleted (very low concentration) status of our SF medium, then verified the essentiality of each factor (except selenium) during skeletal muscle differentiation. To confirm the change in Se status during cell differentiation, cellular Se concentration and glutathione peroxidase (GSH-Px) activity were monitored. We also followed the change in selenoprotein W expression during differentiation. Thus, the SF medium obtained here was used for our future Se studies.

Materials and methods

Materials

L8 rat myoblast cells were obtained from American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle medium (DMEM), DMSO, penicillin/streptomycin solution, trypsin, and fetuin were purchased from GIBCO (Grand Island, NY). Dexamethasone, insulin, and sodium selenite were purchased from Sigma (St. Louis, MO). Linoleic Acid/BSA was obtained from Collaborative Biomedical (Bradford, MA). Transferrin was purchased from Calbiochem (La Jolla, CA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Cell culture dishes were purchased from Corning (Corning, NY).

Cell Culture

Original cell stocks, stored in liquid nitrogen, were thawed and subcultured to obtain large numbers of cells. Cells were aliquoted and again stored in -80°C with 10 % DMSO for later use. Working cells were maintained by repeated subculturing at low density on 10 cm culture dishes. Cells were grown in proliferation medium, DMEM supplemented with 10% FBS, 100 units of penicillin/ml, 100 μg of streptomycin/ml, 44mM $NaHCO_3$, in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. To transfer, cells were removed with 0.25% trypsin in Ca^{2+}- and Mg^{2+}- free Ham's balanced salt solution (HBSS; 5.4 mM KCl, 0.3 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 5.6 mM glucose; pH=7.4) and transferred to 6 or 10 cm culture plates for experiments. Cells were grown in 10% FBS and medium was changed every 2 days until

cells reached confluence. Then, medium was changed to differentiation medium (serum free medium) to induce differentiation.

Serum-Free Medium

The serum-free medium was developed for L8 skeletal muscle cells based on R.E. Allen's (1985) SF medium for skeletal muscle satellite cells. The serum-free medium was composed of DMEM, supplemented with penicillin, streptomycin, and $NaHCO_3$ (at the same concentration used in the proliferation medium), 0.3 mg fetuin/mL (Petersen method), 1 μg linoleic/ml, 10^{-7} M dexamethasone, 10^{-7} M insulin, 5 μg transferrin/ml, plus different concentrations of selenium added as sodium selenite.

The basic protocol for obtaining selenium-deficient cells was as follows:

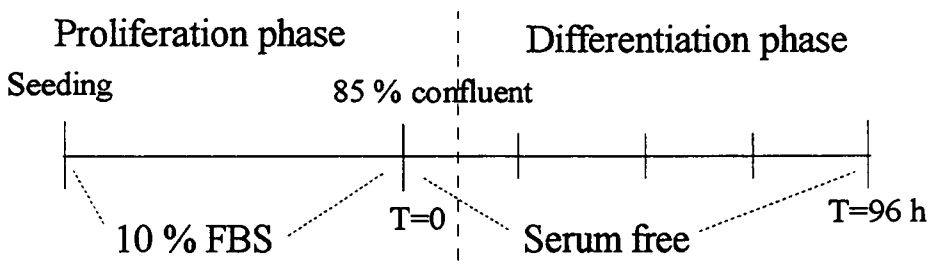

Figure 1. Treatment outline

At Time=0, myoblasts are approximately 85% confluent and media were then changed from proliferation media (DMEM supplemented with 10% FBS) to serum-free media to stimulate differentiation.

To confirm low Se status of this media, Se concentration was determined. Samples (5 ml) of distilled water, DMEM (no additives), SF medium without Se supplement, SF medium with Se supplement (2.5×10^{-7} M), and 2 % horse serum supplemented medium were allocated for Se concentration assay as described in a later section.

Harvesting Cells

When cells were collected following treatments, each plate of cells was washed with ice-cold phosphate-buffered solution (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.4) twice. After this, lysing solution (25 mM Tris pH 7.8, 10 mM KCl, and 1 mM MgCl) was added to each plate (0.5 mL for 6 cm plates, 1 ml for 10 cm plates). Cells were scraped into microtubes and centrifuged at 17,000 ×g for 5 min. at 4°C. Samples were then separated into supernatant and pellets.

Serum-Free Medium Component Assay

The effectiveness of each of the five additives for SF medium was tested by assessing the ability of muscle cells to differentiate when individual SF components were omitted from media. After growing muscle cells to near confluence in serum-containing medium, the medium was switched to serum-free medium of varying compositions (t=0). A medium containing all of the SF components (with a Se concentration of 2.5×10^{-7} M supplemented) was used as a positive control. The other five media consisted of all the ingredients except missing one (insulin, dexamethasone, linoleic acid, fetuin, or transferrin). Cells were continually grown and induced to differentiate for 96 hours, and

fresh medium was provided at 24 and 72 hours. Cells were collected at 96 hours with the harvesting method described above. After the centrifugation, the supernatant portion of the separated sample was used for a creatine kinase (CK) analysis.

Creatine kinase activity was measured by using the Creatine Kinase reagent (Sigma). The main reactants contained in this kit were creatine phosphate, adenosine diphosphate (ADP), D-glucose, hexokinase (HK), and nicotinamide adenine dehydrogenase (NAD). By providing the CK present in myotube supernatant, the following reaction is catalyzed:

$$\text{Creatine Phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{G-6-P}$$

$$\text{G-6-P} + \text{NAD} \xrightarrow{\text{G-6-PDH}} \text{6-PG} + \text{NADH}$$

During the oxidation of NAD to NADH, absorbance of 340 nm changes directly proportional to CK activity.

In a polystyrene cuvette, 1 ml of CK reagent was combined with 0.02 ml of cell samples, inverted three times, and incubated for 3 min. Then, 340 nm light absorption was measured by using a Shimadzu UV-160 spectrophotometer. Incubation was continued for another 2 min. during which three more 340 nm absorption readings were taken at 30 seconds of interval. The CK activity was first calculated in activity unit/ml using the formula provided by Sigma:

$$CK\ (U/L) = \frac{\Delta A \text{ per min.} \times TV \times 1000}{6.22 \times LP \times SV}$$

Where:

ΔA per min. = change in absorbance per minute at 340 nm

TV = Total volume (mL)

SV = Sample volume (mL)

6.22 = Millimolar absorptivity of NADH at 340 nm (extinction coefficient)

LP = light path

1000 = conversion of units per mL to units per liter

One unit of activity is defined as the amount of enzyme which produces one µmole of NADH per minute under the conditions of the assay procedure. Creatine kinase activity was normalized to Unit/mg of soluble proteins.

Assessment of Protein Content

The sample protein concentration was determined by the method developed by Bradford (1976), using bovine serum albumin as a standard. Briefly, 3.4 mL of Protein Assay Dye Reagent Concentrate (Bio-Rad; 1:4 diluted with dH_2O) containing Commassie Brilliant Blue G-250 dye, phosphoric acid, and methanol is added into a polystyrene cuvette. To this, 20 µl of supernatant portion of cell lysate samples were added and mixed

by pipetting twice. After incubation at RT for 10 min, absorbance was measured by spectrophotometer at a wavelength of 595 nm.

Selenium Status Determination

Three methods were used to assess cellular selenium status.

1. Direct selenium assay

Cells were cultured on 150 × 25 mm culture plates in serum-free medium with 0 or 2.5×10^{-7} M Se concentrations added at 85 % confluence. Cells were harvested at various time (0, 24, 48, and 96 hours) and soluble and insoluble portions were separated by centrifugation at 17,000 ×g for 5 min. at 4°C. Selenium content of the supernatant (soluble protein) was assayed by a semi-automated fluorometric method (Brown and Watkinson, 1977) using an Alpchem II system (Alpchem. Corp., Milwaukie, OR). The Se concentration was expressed in ppm per plate of cells. Each samples were measured in duplicate. Selenium level in medium was also determined by this assay.

2. Glutathione Peroxidase (GSH-Px) assay

Cells were treated and collected from 100 mm cell culture plates in the manner described above. After taking 0.1 ml of supernatant and providing 0.8 ml reaction mixture (containing 0.233 mg NADPH, 1.533 mg reduced glutathione, 0.67 units glutathione reductase, and 0.8 ml phosphate buffer containing 59 mM $NaH_2PO_4\cdot7H_2O$, 0.17 M $Na_2H_2PO_4\cdot H_2O$, 4 mM EDTA, and 4.7 mM sodium azide). The enzymatic reaction was initiated by addition of 0.1 mL of 2.2 mM H_2O_2 to the mixture and mixed by inversion three times. Changes in the absorption of 340 nm light was measured for 2 min at

intervals of 30 sec by using Beckman DU series 60 spectrophotometer. Glutathione peroxidase activity was determined by calculating the rate of NADPH oxidation by using the following formula:

$$\text{GSH-Px activity (nm/min.mL)} = \frac{\Delta A \text{ (sample)} - \Delta A \text{ (blank)}}{\text{min x SV}} \times \frac{TV}{6.22}$$

Where:

ΔA = change in absorbance at 340 nm

min = total assay time

SV = sample volume (mL)

TV = total volume (mL)

6.22 = millimolar absorptivity of NADPH at 340 nm (extinction coefficient)

Protein concentration was measured by using Bradford method (1976) and the final GSH-Px activity was expressed in nm/min mg protein.

3. Western blot analysis for selenoprotein W

Cells were cultured on 100 mm plates with 0 or 10^{-7} M added Se. Cells were collected at 0, 24, 48, and 96 hours as described before, using homogenizing buffer (20 mM Tris[pH7.5], 1 mM EGTA, 5 mM β-mercaptoethanol and 25 μg/ml leupeptin) instead of lysing solution, and homogenized using a Dounce homogenizer. After centrifugation for 10 min at 17,000×g, the supernatant were collected and pellets were discarded. Protein content was measured in the supernatant by the Bio-rad assay using bovine serum

albumin as a standard. Samples (150 µl) were electrophoretically separated on SDS-polyacrylamide 7.5 to 15% gradient gels. Proteins were electrophoretically transferred onto nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) with 30 V in 4°C overnight. After transfer, membranes were blocked with 5% non-fat dried milk in TTBS [0.05% Tween 20 in TBS] at room temperature for 1hr, then incubated with rabbit anti-selenoprotein W polyclonal antibody (gift from Dr. P.D. Whanger; Yeh et al., 1995) at room temperature for 1.5 hr. Following three washes with TTBS, membranes were incubated with horse-radish peroxidase-conjugated goat anti-rabbit IgG antibody. Membranes were washed several times with TTBS and TBS and developed with Amersham's ECL detection system. The membranes were then exposed to Kodak X-OMAT film at least 15 minutes.

It must be noted that these experiments were done to establish background data for serum free culture. Therefore, most experiments in this section were not repeated. Thus, statistical analysis was not obtained for these data sets. Results are presented as the means of duplicate or triplicate in one trial.

Results and Discussion

Selenium Concentration in Culture Media

First, we assessed the Se concentration of our serum-free media. Our aim was to obtain the lowest possible selenium level in the extracellular environment. Our serum free medium was found to contain variable but very low Se concentration ($<2 \times 10^{-9}$ to 1.87×10^{-8} M of Se; avg. 1.31×10^{-8} M). Analysis of source water and DMEM (without supplement) revealed that the majority of Se came from DMEM, not from the SF media supplements (Table 1).

Sample	Se-concentration
Distilled H_2O	4.06×10^{-9} M
DMEM (no chemical supplement)	1.45×10^{-8} M
Serum free (no Se supplement, average)	1.37×10^{-8} M
Serum free (Se supplemented)	2.35×10^{-7} M
2 % Horse serum supplemented DMEM	6.17×10^{-8} M

Table 1. Selenium concentrations in cell culture medium.

Although Se was detectable in non-supplemented media, these values were still very low compared to serum Se concentrations. Animal serum selenium levels vary greatly depending on the animal and diet. It can be vary from or 6×10^{-8} M to 5×10^{-7} M (Beilstein et al., 1987).

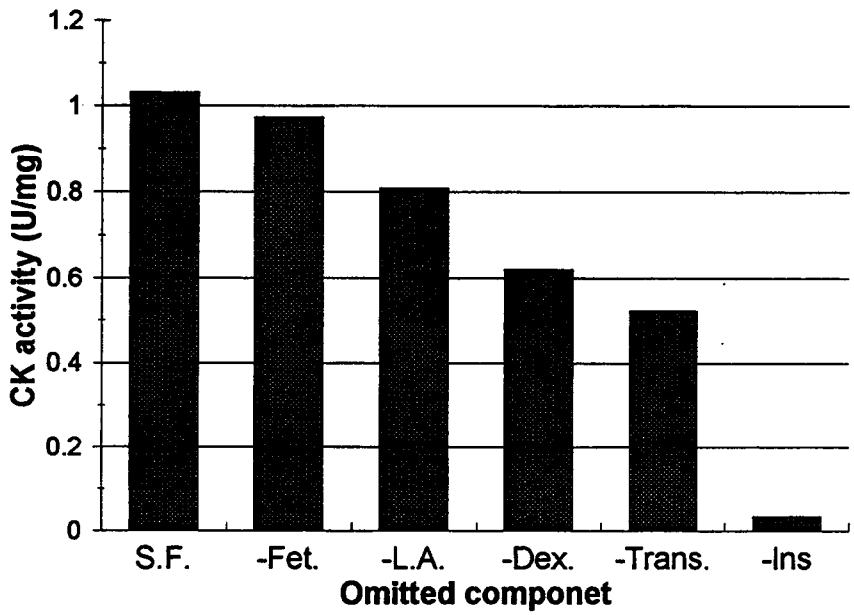

Figure 2 Serum-free medium components were tested by omitting one component at a time. Cells were incubated for 96 hours and differentiation was measured in terms of CK activity. Omitted component is shown on the x-axis. abbreviations: S.F., serum-free medium; Fet, fetuin; L.A., linoleic acid; Dex, dexamethasone; Trans, transferrin; Ins, insulin.

Component Study

The serum-free media used in this study was modified from a SF medium for skeletal muscle satellite cells developed by Dr. R. Allen of University of Arizona (Allen et al., 1985). This media consisted of a 3:1 mixture of DMEM and MCDB-104 as a basal media. To the basal medium, insulin (10^{-6} M), fetuin (0.5 mg/ml), BSA-linoleic acid (1 μg linoleic acid/ml), dexamethasone (10^{-7} M), transferrin (5 mg/ml), fibroblast growth factor (FGF 100 ng/ml), and selenium (3×10^{-8} M) were added. In our preliminary testing for survival of cells in SF medium, MCDB did not show a significant visual effect on survival/differentiation of cells, thus only DMEM was used. Fibroblast growth factor, which did not manifest any increase in survival/differentiation rate under light microscope in the same test, was also omitted before performing the experiments in this study. Thus, by using CK assay, the influence of each serum-free medium component on differentiation was tested (Fig. 2).

The results suggested that most of the components we tested are essential for L8 muscle cell differentiation and adequate support of myotubes. The general aspects of each serum-free component is briefly discussed.

1. Insulin

Insulin has been shown to be an essential factor for growth of most cell lines routinely used in research (Barnes and Sato, 1980b). Insulin withdrawal caused almost complete loss of cellular metabolism such as proliferation (Rechler and Podskalny, 1976), or differentiation in our study (see Fig. 2). We observed that insulin withdrawal increased

loss of myoblasts, indicated by detachment of cells from culture plates. To achieve adequate maintenance of cells and metabolic stimulation in cell culture, insulin concentration had to be extra ordinally high in the serum-free medium compared to what is considered to be a physiological concentration (Barnes and Sato, 1980b). It has been suggested that either rapid inactivation of insulin in basal salt medium, or mimicking of insulin-like growth factors by insulin itself may be causing its high concentration requirement in cell culture (summarized in Barnes and Sato, 1980a).

Insulin is thought to have numerous functions in the cell and all of the roles of insulin are not completely understood. However, as in other tissue types, the regulation of glucose transport has been shown to be the primary role of insulin in skeletal muscle cells (Sarabia et al., 1992). Glucose is an important source of energy to the cells for metabolic activities. Although the precise mechanism of insulin regulation of glucose transport is not clear, it has been shown that binding of insulin to insulin receptor initiates the tyrosine kinase activity which leads to activation of a series of signal transduction pathway reaction inside the cell (Tsakiridis et al.. 1995). It is also suggested that insulin influences muscle differentiation via a signal transduction pathway involving protein phosphatase (Srinivasan and Begum, 1994).

2. Dexamethasone

Although some hormones with a tetracyclic hydrocarbon backbone, such as testosterone and progesterone, have been shown to promote growth of various cell lines, dexamethasone, a glucocorticoid with similar structure, is used only in serum free medium

for muscle cells (Barnes and Sato, 1980b; Allen et al., 1985). In muscle cells, Florini and Ewton (1981) have demonstrated that dexamethasone has more growth stimulatory effect of all glucocorticoid and steroid hormones. Skeletal muscle cell differentiation is also stimulated by addition of 10^{-7} M dexamethasone in serum free media which is almost the same level as normal circulating level of glucocorticoid (Allen et al., 1985). On the other hand, dexamethasone has been shown to interfere with muscle growth and to increase protein degradation at higher concentrations (Dardevet et al., 1995; Hong et al., 1995). In our study, dexamethathone withdrawal led to 40 % decrease in differentiation (Fig. 2), suggesting the importance of dexamethasone to L8 muscle cell culture.

Dexamethasone has been reported to affect several different biochemical aspects of muscle cell metabolism. Insulin resistance induced by dexamethasone in muscle cell causes a decrease in activity of glucose transport (Weinstein et al., 1995). However, since insulin receptor and binding was unaffected by dexamethasone and tissue concentration of glucose transporter does not change, the mechanism by which dexamethasone induces insulin resistance is still unknown (Oda et al., 1995; Saad, 1993). The effect of dexamethasone on protein degradation has been shown to be the result of increased cellular protease activity, both in vivo and in vitro (Dardevet et al., 1995; Hong et al., 1995).

The mechanism of growth and differentiation promotion in muscle cells by dexamethasone is also unclear, however, Giorgino et al.. (1995) have demonstrated that dexamethasone enhanced phosphorylation of IGF-1 receptor tyrosine and phosphatidylinositol 3-kinase activity. Although our serum free medium did not contain

IGF-1, it is possible that dexamethasone enhanced mitogenic activity of muscle cells via stimulation of the insulin signal transduction pathways.

3. Transferrin

As its name implies, the main function of transferrin in the animals is to transport iron to various tissues. Iron is utilized primarily in erythrocytes as an essential component of hemoglobin (Borch-Iohnsen, 1995). However, other roles of iron have been discovered in various tissues (Theil, 1987). For example, iron plays an important role in activity of the enzyme ribonucleotide reductase. Activity of this enzyme is very critical during DNA synthesis (Reichard and Ehrenberg, 1983).

It has been reported that transferrin, like insulin, is required in serum-free media for most cell lines (Barnes and Sato, 1980b), except L6 rat skeletal muscle cells (Florini and Roberts, 1979). In fact, Allen et al. (1985) concluded that transferrin was not necessary in serum-free medium to stimulate rat satellite cell differentiation. However, they also mentioned the presence of $FeSO_4$ in MCDB-104 basal medium might provide sufficient iron to stimulate cellular metabolism. $FeSO_4$ had a similar effect as transferrin in serum-free medium in other types of cells (Mather, 1979).

Despite these two findings, we found that lack of transferrin in serum free medium caused a 50 % reduction in differentiation (Fig.2). It is possible that $FeSO_4$ could replace transferrin even in L8 cells. However, since transferrin is relatively easy to supplement and we had no information concerning supplementation of $FeSO_4$ in muscle cell culture, transferrin was continually used in this study. It maybe useful comparing these two forms

of iron sources for the future references. Also, investigation of the presence of a transferrin receptor on both L6 and L8 cells could reveal difference in transferrin susceptibility between these two cell lines, or may even identify distinct differences in muscle from other tissues.

4. Linoleic acid

Linoleic acid is an essential fatty acid (EFA) which is necessary to maintain normal tissue and membrane function (Houtsmuller and Beele, 1981), and is a precursor for hormones such as prostaglandin and thromboxane (Mathews and Holde, 1990). By contrast, these are not the primary functions of linoleic acids in cultured tissue cells. Some cell lines can grow normally in the absence of any EFA (reviewed by Rosenthal, 1987).

Stimulation of proliferation has been reported to be the major response of cultured cells to linoleic acid (Keller et al., 1992; Bandyopadhyay et al., 1987). Gadiparthi et al. (1995) found that linoleic acid increased DNA synthesis and growth-response gene (such as c-fos and c-jun) mRNA expression. The role of linoleic acid in promoting skeletal muscle cell differentiation is unknown (Allen et al., 1985). However, Allen et al. (1985) found linoleic acid to be the most critical factor in their SF media for stimulating cell fusion during differentiation of skeletal muscle satellite cells. By omitting linoleic acid, there was approximately a 90 % reduction in cell fusion rate.

In our study, we found a 22 % reduction in differentiation compared to complete SF media after 96 hours of incubation without linoleic acid (Fig. 2). Although the

reduction was not as critical as that was found by Allen et al. (1985), the result suggests the importance of linoleic acid to differentiation of L8 skeletal muscle cells.

The linoleic acid used in this study was complexed with bovine serum albumin (BSA). The main role of BSA is to transport linoleic acid into the cells. Also, if there is any unbound BSA generated, it may has a role of transporting other molecules. It is also suggested that BSA has a "detoxifying effect" in the medium (Barnes and Sato, 1980a).

5. Fetuin

Fetuin was initially identified as an α-globulin in fetal calf serum (Pedersen, 1944). Fetuin is a major component of serum and it was found to have a growth-stimulating effect in many cell lines (reviewed by Nie, 1992). However, it was later found out that fetuin prepared by Pedersen was rather crude and several minor contaminants were detected by gel electrophoresis. Further purification of fetuin by Deutch (1954) and Spiro (1960) caused the inability of fetuin to stimulate growth of most cell lines except muscle cells (Nie, 1992). It has been reported that one of the fetuin contaminants promoted partial growth of rat kidney and mammary epithelial cells (Salomon, 1982). Considering these results, use of fetuin in our SF medium was controversial.

Even in skeletal muscle cell line, fetuin was found to be effective only when it was used in place of transferrin (Allen et al., 1985; Florini and Roberts, 1979). Our study showed that fetuin had the least effect on promoting cell differentiation (Fig. 2), and our medium does contain transferrin. It is possible that iron contamination of fetuin may

account for its ability to promote differentiation. However, it is also possible that transferrin and fetuin have common contaminants that promote differentiation.

Since there are other contaminants which not only promote cell differentiation, but may be needed to maintain cell integrity (Nie, 1992), we decided to include fetuin in our SF medium. There is no evidence that selenium is a contaminant of fetuin. Thus, the use of crude fetuin (Pedersen preparation) in our study should be acceptable.

Other Notes about Serum-Free Media for L8 Cells

As can be seen with the possible interactions between insulin and dexamethasone, and transferrin and fetuin, there are complex interactions between each of the components that make- up the SF medium. Therefore, it is desirable to use a SF medium to control the extracellular environment by introducing a limited number of factors. However, at the same time, a disadvantage of SF culture is that it eliminates possible critical interactions between the molecule of interest (Se) and a serum component which may be present serum containing media.

We have developed a serum free medium which supports L8 muscle cell differentiation. However, we were unable to develop a SF media which would promote proliferation of L8 cells. Addition of factors such as EGF or FGF to SF media did not stimulate or permit proliferation. Also, four different commercially prepared serum free media for non-muscle cells (Cellgro, Mediatech Inc.; UltraCulture, BioWhittaker; Endothelial SFM, Gibco; and HyQ, HyClone) were unable to promote proliferation.

Thus, in this study, cells were only treated with Se-deficient SF medium after reaching confluence.

Effect of Se-Free Medium on Cellular Selenium Concentrations

To assay the efficiency of our low-Se serum-free media in reducing cellular Se, we assessed cellular Se concentration at varying times following the switch to SF media. Changes in selenium concentration in the soluble portions (supernatant) of cells were assayed (Fig. 3). It must be noted that cells were incubated with 10 % FBS until 85 % confluence, or t=0. Thus, the cells already contained an adequate level of selenium at the beginning of the assay (t=0).

Selenium concentration clearly declined until 48 hours after changing to low selenium medium, then stabilized until 96 hours (Fig. 3). Retention of selenium in the cells after 48 hours, could result from integration in proteins that have a slow turn over rate, or from incorporation of the minimal selenium in serum free medium. On the other hand, cells supplemented with adequate Se increased in selenium concentration during the first in the 24 hours, and then maintained relatively stable Se concentrations up to 96 hours. Overall, cellular selenium content was responsive to the different media selenium levels.

Glutathione Peroxidase (GSH-Px) Activity

GSH-Px is known to contain selenium in its active site (Epp et al., 1983). Therefore, when cells are deprived of selenium, the amount of GSH-Px should also decrease in the cells. Thus, GSH-Px represents a functional measure of Se status in the cell. GSH-Px is so far the most useful selenoenzyme to assay biopotency of selenium in

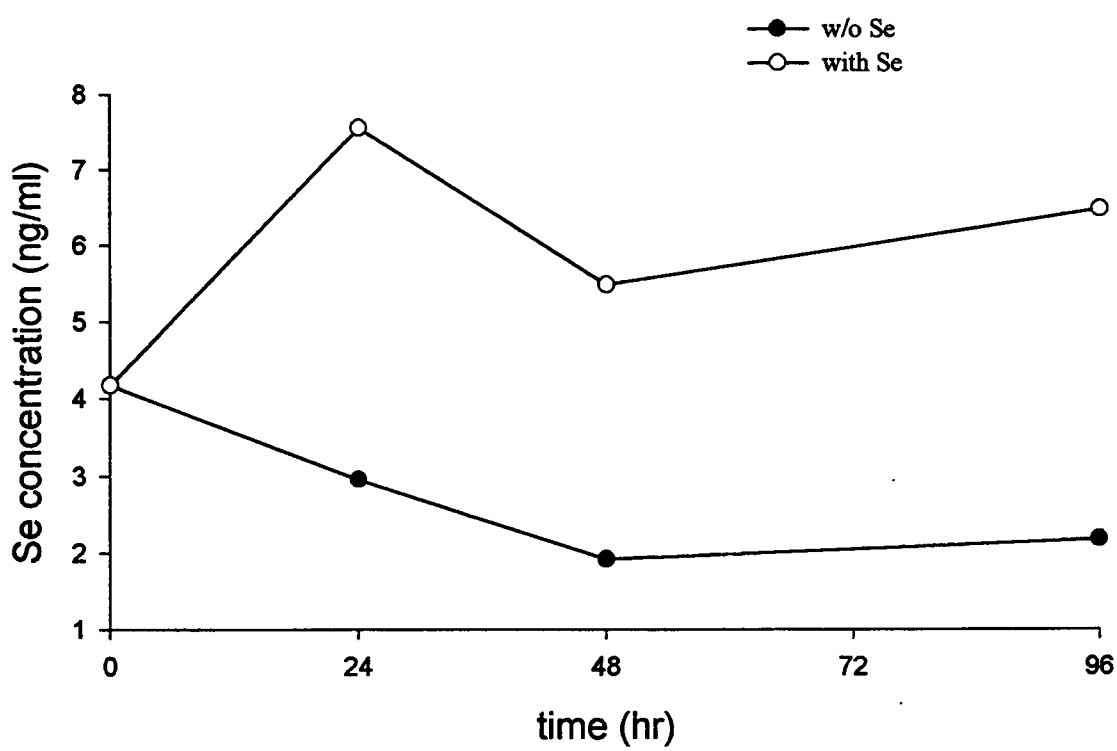

Figure 3. Effect of serum-free medium on Se concentration of L8 muscle cells. Myoblasts were incubated with 0 or 2.5 x 10^{-7} M selenium for 96 hours. Se concentration in soluble fraction was measured (ng/ml). Samples were collected at 0, 24, 48, and 96 hours after the treatment.

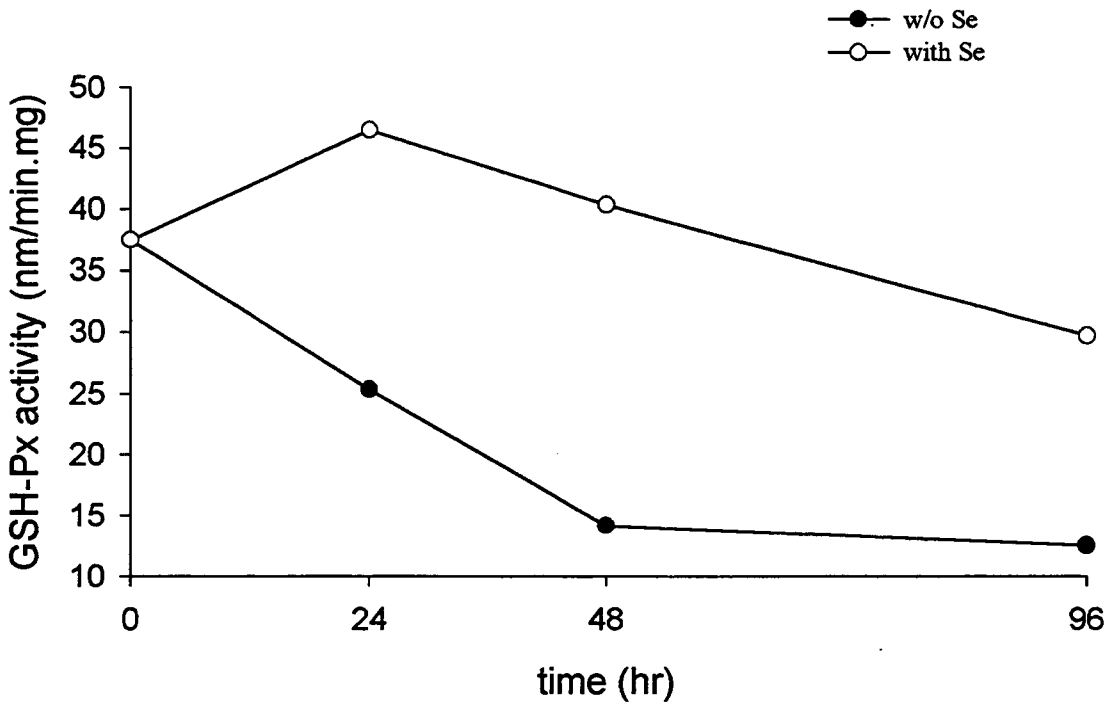

Figure 4. Glutathione peroxidase activity in L8 muscle cells. Myotubes were incubated with serum-free media with 0 or 2.5 x 10^{-7} M selenium for 96 hours. Samples were collected at 0, 24, 48, and 96 hours after the treatment. GSH-Px is expressed in (nm/min.mg).

animals (Sunde and Hoekstra, 1980; Vendeland et al., 1992; Shan and Davis, 1994; Podoll et al., 1992). Different selenium treatments caused change in GSH-Px activity over time (Fig. 4). Without selenium supplementation, GSH-Px decreased 70 % within 48 hours then stabilized. This was similar to the change in cellular selenium content in Se-deficient muscle cells (Fig. 3). Se-supplementation caused a 30 % increase in GSH-Px activity in the first 24 hours, after which activity decreased near original levels. This increase in GSH-Px activity during L8 cell differentiation was observed previously by Yeh et al. (1997) using 2 % equine serum to stimulate differentiation. Thus, increase in GSH-Px activity may be due to the response to differentiation and not to a change in Se status of the media. Therefore, the difference in GSH-Px activity was greatest following 48 hours of incubation. The difference (>30 %) was continually present until 96 hours of incubation. From these data, it is clear that withdrawal of Se from culture media results in a loss of GSH-Px activity, suggesting the Se-deficient status of the cells.

Selenoprotein W

Selenoprotein W concentration was assessed to provide another means of determining the effects of Se-depletion on cellular Se status. To accomplish this study, a polyclonal antibody against rat selenoprotein W was provided by Dr. Phil Whanger.

Selenoprotein W was not detectable at t=0 (Fig. 5). In cells with no selenium supplementation, selenoprotein W was undetectable throughout the 96 hours of incubation. In contrast, in Se supplemented cells, it was detected at 24 hours of incubation and after.

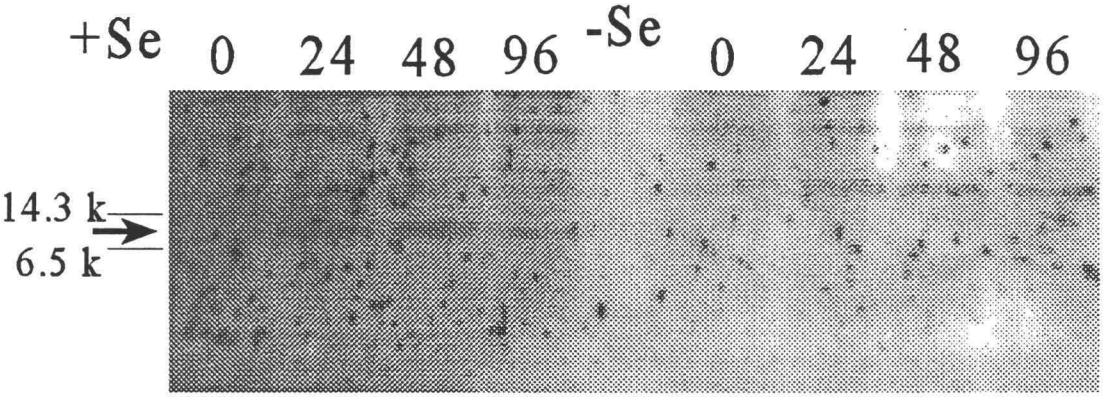

Figure 5. Western blot against selenoprotein W. Cells were grown in serum free medium either with or without selenium for 96 hours. Selenoprotein W is indicated by arrow (approx. 9.5 kDa). All other bands are non specific binding.

Conclusion

We established that we could obtain low Se status in muscle cell culture using serum free medium. Cellular Se concentration assay, GSH-Px activity assay, and Selenoprotein W immunoblotting showed that difference in Se deficiency was observable after 24 hours of Se withdrawal. Although cellular Se was not completely depleted by Se-deficient media, the reduction in Se status was clearly evident. Thus, using this SF medium, we examined the effect of Se deficiency on skeletal muscle cell metabolism.

Chapter 3. Effect of Selenium Deficiency on Muscle Cells

Although the clinical pathology and histology of WMD have been well characterized (Maas, 1996), understanding of biochemical aspects of the etiology of this disease has been very limited (Tripp, 1993). To develop Se-deficient animal is time consuming, and obtaining and processing tissue samples in a timely manner from such animals are sometimes labor intensive. Thus, in addition to the data obtained from in vivo experiments, use of a cell culture system is a useful research tool to further investigate WMD.

L8 rat skeletal muscle cell culture has been routinely used for various molecular and biochemical studies of muscle tissues (Hong et al., 1995; Barnoy et al., 1996). The same cell lines were recently used for studying the effect of Se on selenoprotein W in muscle tissues by Yeh et. al. (in press). Since all cells come from a single clone of original cells, the experimental results are usually consistent. Thus, the L8 rat skeletal muscle cell line was determined to be the most desirable cell line to investigate the biochemical aspects of Se-deficiency in skeletal muscle cells in this study.

In a previous chapter, we have established that we can generate low cellular Se status in L8 cells by using serum-free (SF) media. This medium allowed L8 cells to differentiate and to maintain normal myotube morphology. Thus, in this study, the effects of low Se level on skeletal muscle cell differentiation and protein degradation were studied. Following this we studied the effects of Se-deficiency on cell detachment.

In many WMD studies, vitamin E has been supplemented in the presence and in the absence of selenium (Whanger et al., 1977; Schubert et al., 1961). Vitamin E protects lambs from developing Se-deficient disease completely if it was implanted directly to the lambs, and provided partial protection to lambs if vitamin E was implanted in ewes before parturition (Hidiroglow et al., 1972). Therefore, in our study, we also investigated the effect of vitamin E in preventing the changes in muscle cell physiology by Se-deficiency.

Materials and Methods

Materials

L8 rat myoblast cells were obtained from American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle medium (DMEM), penicillin/streptomycin solution, trypsin, and fetuin were purchased from GIBCO (Grand Island, NY). Dexamethasone, α-tocopherol, insulin, selenium and the creatine kinase assay kit were purchased from Sigma (St. Louis, MO). Linoleic Acid/BSA was obtained from Collaborative Biomedical (Bradford, MA). Transferrin was purchased from Calbiochem (La Jolla, CA). Fetal Bovine serum (FBS) was purchased from Hyclone (Logan, UT). ^{3}H-tyrosine was obtained from DuPont NEN (Boston, MA). Liquid scintillation counting solution was obtained from ICN Biomedicals (Costa Mesa, CA). Corning cell culture petri dishes were purchased from Fisher Scientific (Santa Clara, CA).

Cell Culture

Original L8 cells, stored in liquid nitrogen, were thawed and sub-cultured to obtain a large numbers of cells. Cells were aliquoted and again stored at -80°C with 10 % DMSO for later use. Working cells were maintained by repeated subculturing at low density on 10 cm culture dishes. Cells were grown in DMEM supplemented with 10% FBS, 100 units of penicillin/ml, 100 μg of streptomycin/ml, 44 mM $NaHCO_3$ in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cells were removed with 0.25% trypsin in Ca^{2+}- and Mg^{2+}- free Ham's balanced salt solution (HBSS; 5.4 mM KCl, 0.3 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 5.6 mM glucose; pH=7.4) and

transferred to 6 or 10 cm culture plates for experiments. To induce differentiation, cells were grown in DMEM supplemented with 10% FBS until cells were near confluence. Then, medium was changed to differentiation medium (serum free medium) to induce differentiation.

Serum-Free Medium

The serum-free medium was developed for L8 skeletal muscle cells based on R.E. Allen's (1985) SF medium for skeletal muscle satellite cells. The serum-free medium was composed of DMEM, supplemented with penicillin, streptomycin, and $NaHCO_3$ (at the same concentration used in the proliferation medium), 0.3 mg fetuin/mL (Petersen method), 1 µg linoleic/ml, 10^{-7} M dexamethasone, 10^{-7} M insulin, 5 µg transferrin/ml, plus different concentrations of selenium added as sodium selenite. When myoblasts are approximately 85% confluent and media were then changed from proliferation media (DMEM supplemented with 10% FBS) to serum-free media to stimulate differentiation.

Creatine Kinase (CK) Assay

After cells reached 80 % confluence in 6 cm culture plates, medium was changed to serum-free (t=0) with Se added at either 0 or 2.5×10^{-7} M. Cells were harvested at 0, 24, 48, and 96 hr after the treatment. Immediately before harvesting, each plate of cells was washed twice with ice-cold phosphate-buffered solution (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.4). To lyse cells, 0.5 mL lysing solution (25 mM Tris pH 7.8, 10 mM KCl, and 1 mM MgCl) was added to each plate and cells were scraped into microtubes and centrifuged at 17,000 ×g for 5 min. at 4°C.

Sample was then separated into supernatant and pellet fractions and stored at -20°C until assayed. Sample supernatants, containing soluble protein, were then assayed for CK activity.

Creatine kinase activity was measured by using the Creatine Kinase kit (Sigma). In a polystyrene cuvette, 1 ml of CK reagent was combined with 0.02 ml of cell samples, inverted three times, and incubated for 3 min. Then, 340 nm light absorption readings were taken by using a Shimadzu UV-160 spectrophotometer. Incubation was continued for another 2 min. during which three more readings at 340 nm absorption were taken at 30 second intervals. The CK activity was first calculated in unit/ml using the formula provided from Sigma:

$$\text{CK (U/L)} = \frac{\Delta A \text{ per min.} \times TV \times 1000}{6.22 \times LP \times SV}$$

Where:

ΔA per min. = change in absorbance per minute at 340 nm

TV = Total volume (mL)

SV = Sample volume (mL)

6.22 = Millimolar absorptivity of NADH at 340 nm (extinction coefficient)

LP = light path

1000 = conversion of units per mL to units per liter

One unit of activity is defined as the amount of enzyme which produces one μmole of NADH per minute under the conditions of the assay procedure.

The sample protein concentration was determined by the method based on the Bradford assay (1976; see below). Finally, creatine kinase activity was normalized to Unit/mg of soluble proteins.

Assessment of Protein Content

The sample protein concentration was determined by the method developed by Bradford (1976), using bovine serum albumin as a standard. Briefly, 3.4 mL of Protein Assay Dye Reagent Concentrate (Bio-Rad; 1:4 diluted with dH_2O) containing Commassie Brilliant Blue G-250 dye, phosphoric acid, and methanol was added to a polystyrene cuvette. To this, 20 µl of supernatant portion of cell lysate samples were added and mixed by pipetting twice. After incubation at RT for 10 min, absorbance was measured by spectrophotometer at a wavelength of 595 nm.

Assaying of Total Protein Degradation

Cells in 35 mm culture dishes were incubated for 96 hours with serum-free medium without Se to induce differentiation. Fresh media was provided at 24 and 72 hours. At 96 hours, 0.5 µCi/ml ^{3}H-tyrosine was provided to the cells in 2 ml fresh SF medium for 24 hours. At this point (t=0), the ^{3}H-tyrosine containing medium was discarded and replaced with 1.6 ml SF medium containing 2 mM tyrosine and selenium at various concentrations (0, $2.5x10^{-7}$ M, or $2.5x10^{-6}$ M). Cells were incubated, then media was collected at 0, 24, and 48 hours. Media samples were treated with 10 % TCA overnight at 4°C to precipitate protein. After centrifugation at 17,000 g for 5 min., supernatant was transferred to counting vials with 8 ml of liquid scintillation counting

solution. Radioactivities of the samples were determined by Beckman Model LS6000 SE scintillation counter. Since entire medium from each plate is counted for radioactivity, protein degradation is expressed in radioactivity released (cpm)/plate.

Index of Cell Detachment (Cell Death)

TCA precipitated pellets from the ^{3}H-tyrosine labeling method were dissolved in 1 mL solution of 0.5 N NaOH and 0.1 % Triton X-100. Samples were then transferred into a counting vial and 5 mL liquid scintillation counting solution was added. Radioactivity was the counted by liquid scintillation counting. Since the entire TCA precipitated pellets were counted for radioactivity, cell detachment is expressed in cpm/plate.

Vitamin E Studies

1. Differentiation study

Cells were incubated in DMEM containing 10 % FBS medium on 6 cm plates until 85 % confluence (t=0). The medium was then changed to SF differentiation medium with various levels of vitamin E (0, 10, 35, or 100 μM) and with 2 different concentrations of Se (0 or 2.5×10^{-7} M). Cells were continuously incubated and fresh medium was added at 24 and 76 hours. At 96 hours, all cells were washed twice with PBS and harvested with 0.5 mL lysing solution. Creatine kinase activity was measured by using the Creatine Kinase reagent (Sigma). In a polystyrene cuvette, 1 ml of CK reagent was combined with 0.2 ml of cell samples, inverted three times, and incubated for 3 min. Then, light absorption at 340 nm was measured by using a Shimadzu UV-160 spectrophotometer.

Incubation was continued for another 2 min. during which three more readings at 340 nm were taken at 30 second intervals. Creatine Kinase activity was first calculated in Unit/ml using the formula provided from Sigma (see "Creatine Kinase Assay" section), then normalized to Unit/mg of soluble protein.

2. Cell detachment study

Cells were incubated in 35 mm culture dishes in DMEM supplemented with 10 % FBS. When confluent, differentiation was induced by addition of serum-free medium without Se during a 96 hr incubation. During that time, medium was changed at 24 and 72 hours. Cells were then labeled with 0.5 μCi/ml ^{3}H-tyrosine in 2ml fresh SF media for 24 hours. Cells were then treated with two levels of Se (0 or 2.5×10^{-7} M), and various levels of vitamin E (α-tocophenol; 0, 10, 35, and 100 μM). After 48 hours of incubation, medium was collected and protein released into the media were precipitated by addition of trichloroacetic acid (TCA; 10 % final concentration w/v) and incubated for overnight. After centrifugation at 17,000 g for 5 min., pellets were transferred separate counting vials with liquid scintillation counting solution of 8 and 5 ml, respectively. Radioactivities of the samples were determined by Beckman Model LS6000 SE scintillation counter. Since the entire TCA-precipitated pellet from each plate was counted for radioactivity, cell detachment was expressed as cpm/plate.

Statistical Analysis

In all experiments, cells were randomly assigned to a treatment as a group. All experiments were repeated at least three times with at least two replicates for each

treatment. Data were analyzed with multiple way ANOVA with the Fisher's least-significant difference (LSD) method for comparing groups using StatPlus (v. 2.0 for Windows). A significance level of $p<0.05$ was adopted for all comparisons.

Results

Effects of Se on Cell Differentiation

To assess the hypothesis that Se deficiency results in a reduction in the rate of muscle cell differentiation, we assessed the effect of Se concentration on expression of CK activity (Fig. 6). In all studies, cells exposed to a Se level of 2.5×10^{-7} M are referred to as the positive control.

Se-depletion did not cause a significant change in cell differentiation up to 48 hours ($p>0.05$). However, differentiation rate was significantly decreased at 96 hours after the low Se treatment compared to Se-sufficient cells ($p<0.05$). Selenium-deficiency did not completely inhibit cell differentiation, but only slowed differentiation. This study initially included a Se level of 2.5×10^{-6} M along with the other two levels (0 and 2.5×10^{-7} M). However, this concentration caused cellular death within 48 hours. Thus, it was not used for repeated studies.

Effects of Se on Protein Degradation Rate

To assess the hypotheses that Se deficiency causes muscle atrophy by stimulating muscle protein degradation, we assessed effects of Se deficiency *in vitro* in total protein degradation. Protein degradation rate was estimated by measuring ^{3}H-radiolabelled free amino acid secreted into media. Radioactivity was expressed as cpm/plate (Fig. 7). Cellular degradation was not affected at 24 or 48 hours in any selenium (0, 2.5×10^{-7}, and 2.5×10^{-6} M) treatments ($p>0.05$). Because of the experimental condition using radioisotopes, viability of cells beyond 48 hours was very limited. Thus, both protein

degradation and cell detachment studies were conducted 48 hours of treatment. During the course of this study, it became appearent that more cells were detaching and probably dying when incubated in the absence of Se. We believed that this loss of cells could be the bases of muscle atrophy *in vivo* and therefore decided to assess effects of Se on cell detachment and death.

Effects of Se on Cell Detachment

Effects of medium Se concentration on cell detachment were assayed by measuring TCA-precipitated protein from medium. An increase in TCA-precipitated protein indicates an increase in detached cells, most likely dead cells. Radioactivity was expressed as cpm/plate (Fig. 8).

The difference in detachment was not significant at 24 hours of incubation with the treatment ($p>0.05$). However, at 48 hours, there was a significant increase (40 %) in detachment of cells in the Se-depleted group, and the other two treatment groups (2.5×10^{-7} and 2.5×10^{-6} M) had lower cell detachment with similar values ($p<0.05$, multiple range test). Therefore, this result indicates Se-deficiency cause increased cell death of myotubes.

Effects of Vitamin E Treatment on Differentiation Rate

Since vitamin E and Se have similar and overlapping functions, our next goal was to assess whether the effects of Se on differentiation could be obviated by addition of vitamine E as α-tocopherol (dissolved in 1.9 % ethanol, to culuture media. To simplify the experiment, only one time point (96 hours of treatment) was used in this experiment.

In this study (Fig. 9; Table 2), Se-depletion once again reduced CK activity. This indicates Se is required for optimal rates of differentiation. Vitamin E, in the presence or absence of Se, did not increase CK activity. However, it is interesting to note that Se-depletion caused a significant decrease of CK activity in absence or with lower (10 and 35 uM) vitamin E concentration ($p<0.05$), but did not significantly decrease CK in the presence of high vitamin E concentration (100 uM; $p>0.05$). These results suggest that a high concentration of vitamin E could reduce the impact of Se deficiency in muscle cells.

Effects of Vitamin E Treatment on Cell Detachment

To assess the effect of vitamin E in compensating for the effects of Se deficiency on cell detachment, cells were treated with various concentrations of vitamin E (0, 10, 35 and 100 μM) and Se treatments of either 0 or 2.5×10^{-7} M. The results for both experiments did not show any significance by treatments ($p>0.05$, data not shown). Thus, vitamin E is not effective in rescuing L8 rat skeletal muscle cells from cell detatchment caused by Se-deficiency.

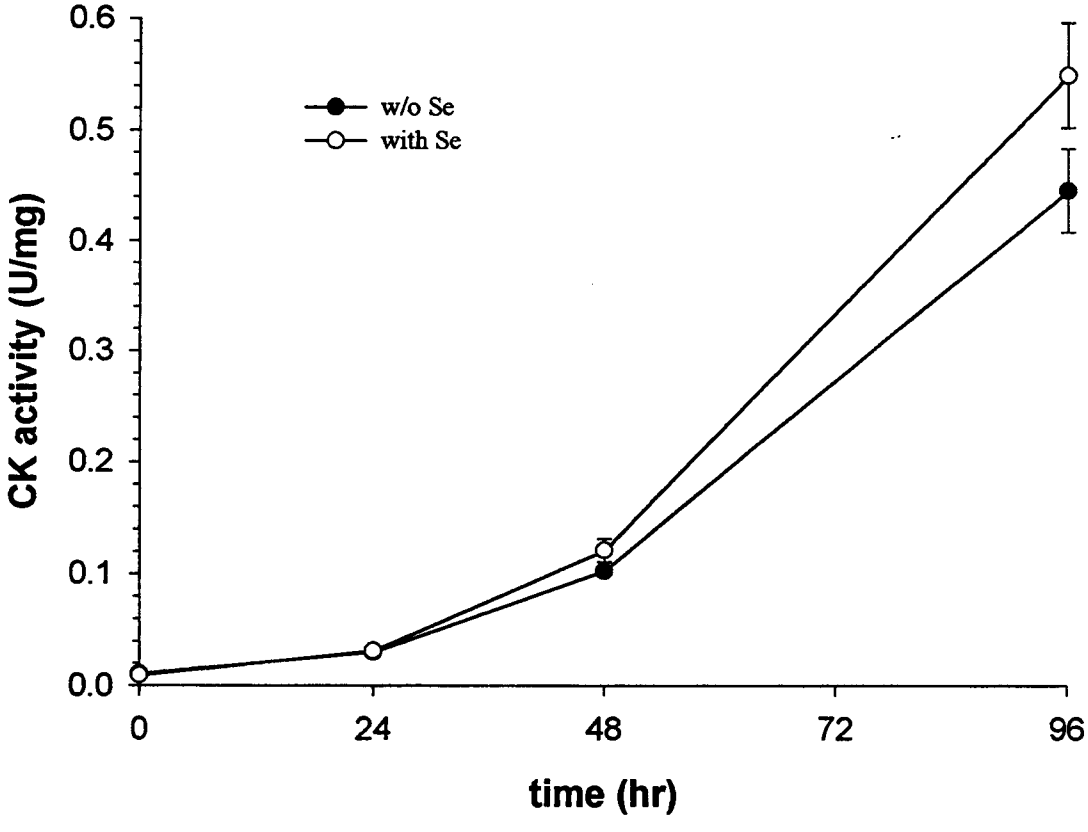

Figure 6. Effects of Se on differentiation rate of L8 muscle cells. Confluent myotubes were incubated in serum-free medium with 0 or 2.5 x 10^{-7} M selenium. Cells were collected at 0, 24, 48, and 96 hours of incubation. Differentiation was assayed in terms of creatinine kinase activity (U/mg). Error bars indicate standard error of mean of repeated trial averages.

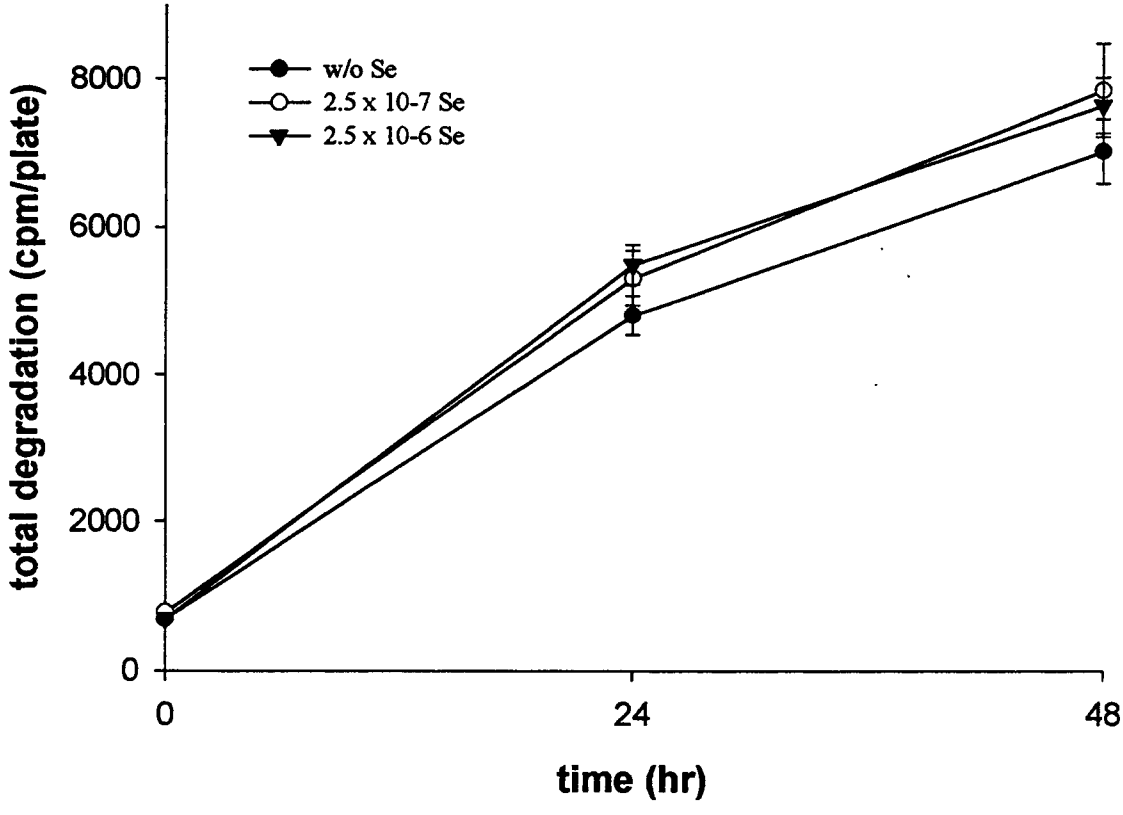

Figure 7. Effects of Se on protein degradation in L8 muscle cells. After 96 hours of Se deprivation, cells were radiolabelled with ^{3}H-tyrosine for 24 hours. Cells were then incubated with 0, 2.5 x 10^{-7}, or 2.5 x 10^{-6} M selenium. Medium was collected at designated times (0, 24, and 48 hours) and radioactivity of the non-TCA-precipitable portion was counted. Radioacitivity is expressed in cpm/plate. Error bars indicate standard error of mean of repeated trial averages.

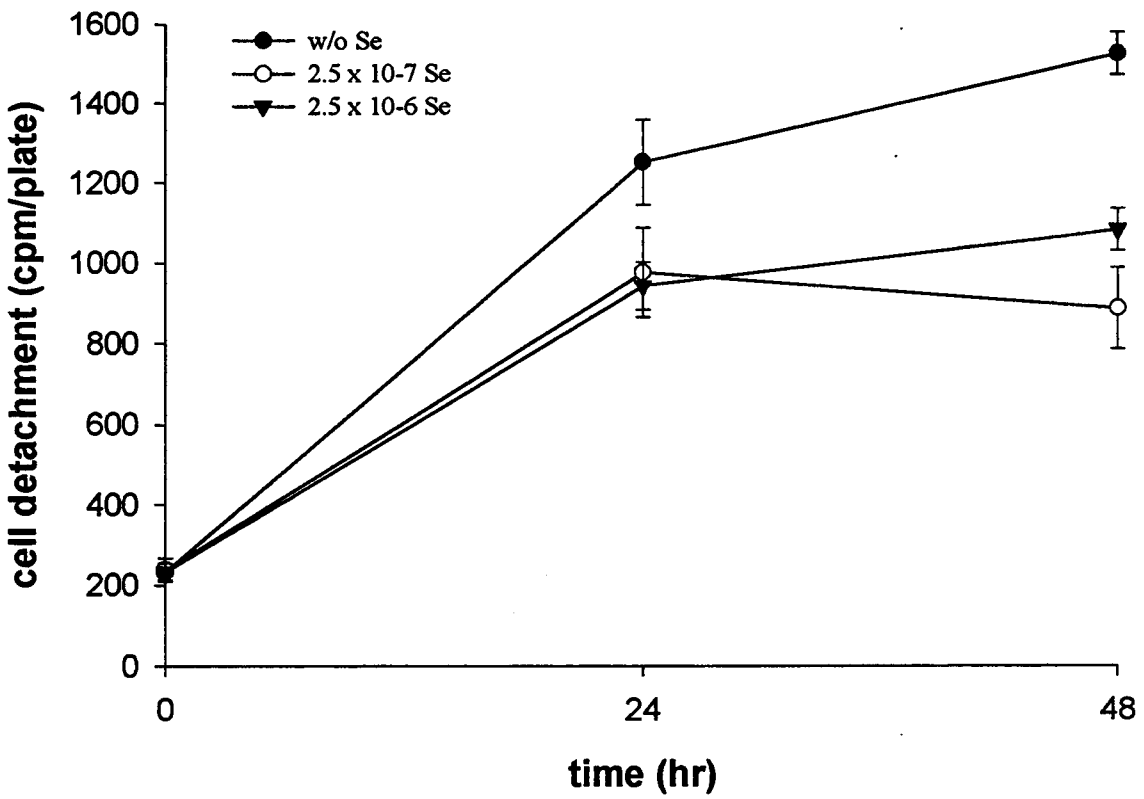

Figure 8. Effects of Se on cell detachment from culture plates.
After 96 hours of Se deprivation, cells were radiolabelled with ^{3}H-tyrosine for 24 hours. Cells were then incubated with 0, 2.5 x 10^{-7}, or 2.5 x 10^{-6} M selenium. Medium was collected at designated times (0, 24, and 48 hours) and radioactivity of the TCA-precipitable portion was counted. Radioactivity is expressed in cpm/plate. Error bars indicate standard error of mean of repeated trial averages.

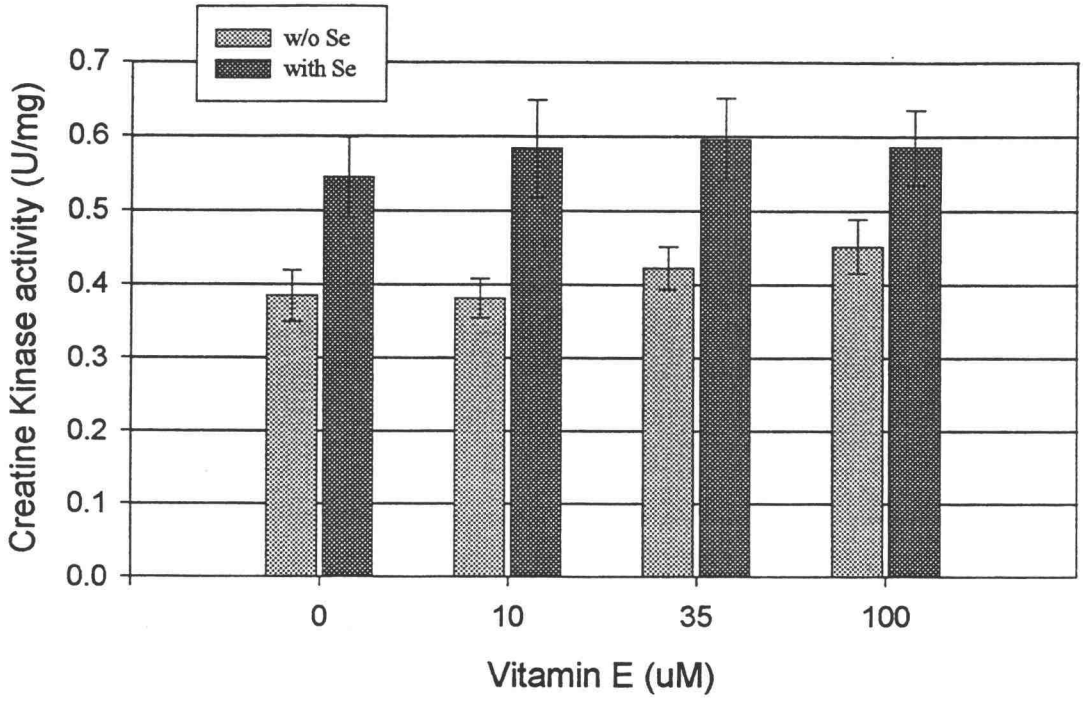

Figure 9. Interaction of Se and vitamin E in control of differentiation of cultured muscle cells. Confluent myoblasts were incubated in serum-free medium with ($2.5x10^{-7}$ M) or witout Se, and various levels of Vitamin E (0, 10, 35, and 100 uM). Cells were collected after 96 hours of incubation and assayed for creatine kinase activity (U/mg). Error bars indicate standard error of mean of repeated trial averages.

Table 2. Effects of vitamin E and Se on differentiation

Creatinine Kinase activity (U/mg)[1]

Vitamin E (μM)	without Se	standard error	2.5×10^{-7} M Se	standard error
0	0.3842 ^{a}	±0.03509	0.5449 ^{bc}	±0.0535
10	0.3813 ^{a}	±0.02672	0.5863 ^{c}	±0.6510
35	0.4227 ^{ab}	±0.02931	0.5956 ^{c}	±0.05478
100	0.4520 ^{abc}	±0.03653	0.5852 ^{c}	±0.04983

1- means which lack a common superscript differ significantly ($p<.05$)

Discussion

Young, rapidly growing lambs and calves are susceptible to white muscle disease (WMD). To induce WMD, ewes and cows have to be selenium-deficient during pregnancy. In experimental situations, these animals need to be fed Se deficient diets before and throughout pregnancy. However, Young et al. (1961) suggested that the late gestation period and early lactation are the most critical times for the mother to receive adequate amounts of Se to prevent WMD. Hartley (1963) suggested that the few months prior to breeding are the critical periods for mothers to receive Se supplementation. This is mainly to assure fertility; however, incidence of WMD was also increased in lambs born to the ewes which did not receive Se during this period. These and other studies suggested the presence of two different forms of WMD, called congenital and delayed white muscle disease (Hungerford, 1990). In the congenital form of WMD, heart muscle is mainly affected starting a few weeks prior to birth, whereas the delayed form shows clinical signs of stiffness in skeletal muscle a few weeks after birth (Hungerford, 1990). Thus, the timing of Se supplementation to ewes has been suggested to have a role in deciding which form of WMD may occur (Hartley, 1963). To explain these observations, Se deficiency may affect various developmental stages of muscle development. In support of this, our experiments showed that Se deficiency can affect both differentiating and fully developed L8 rat skeletal muscle cells.

Se-deficiency of Muscle Cells

Se deficiency caused an approximate 15 % reduction in L8 cell differentiation. Although the relationship between selenium and muscle differentiation has not been elucidated, Se as in antioxidant is most likely to have a role. Se deficiency may cause loss of myoblasts or may impair myoblast physiology due to oxidative damage, thus causing fewer cells to undergo differentiation. This is supported by the fact that GSH-Px, an antioxidant, level decreased within 48 hours in Se-deficient cells. Free radical agents such as H_2O_2 are generated during cell metabolism. Therefore, cells undergoing differentiation could be producing free radicals and Se- deficient cells with less GSH-Px may be more susceptible to oxidative damage. An alternative is that Se may be directly required for the process of myogenesis to proceed optimally. Although we do not have direct evidence of this, it is possible that Se deficiency influences the process of myogenesis. Thus, very low Se concentrations in Se-deficient cells may have reduced the rate at which myogenesis occurred.

Although protein degradation was unaffected by Se deficiency, there was a significant increase in cell death (detachment) of Se-deficient cells. Specifically, cell detachment was increased 65 % ($p<0.05$) following 48 hours of incubation in Se-deficient media. This confirms the Se requirement for maintaining myotubes. Cells were already Se-deficient at the time of Se treatment ($t=0$). Therefore, our results suggest that supplementation of Se will help preventing muscle cell death even after cells became Se deficient. This is in agreement with the fact that, up to certain severity, WMD is reversible by Se-injection (Maas, 1990).

We do not know exactly which selenoproteins are involved in protecting muscle cells against cell detachment (death). Our data suggest that the supplemented Se was rapidly incorporated into the cell to provide protection (within 48 hours). The selenoprotein prevents membrane damage, one of the primary pathophysiology of WMD (Tripp et al., 1993; Yu-zhen et al., 1993). Oxidative damage due to the decreased GSH-Px activity is suggested to be the main cause of the loss of membrane integrity. However, cellular GSH-Px activity has been shown to raise rather slowly. Thus, it does not correlate with the rapid response of WMD animals to Se injection (Maas, 1993). Although it has never been investigated, phospholipid GSH-Px (PHGPX) could have a more important role protecting muscle cell membranes, since that is where this enzyme have been shown to localize. Selenoprotein W could also be an important selenoprotein, since changes in its concentration correlate with the Se status (Vendeland, 1995).

Vitamin E

Vitamin E has been known to effectively protect Se-deficient animals from developing WMD, if it was given directly to lambs (Oldfield et al., 1960). Treatment of vitamin E to ewes prenatally has been shown to provide partial to full protection to the lambs (Schubert et al., 1961; Hidiroglow et al., 1972). Vitamin E is an antioxidant and protects cells from oxidative damage as GSH-Px does. However, it is most effective when Vitamin E was administered with Se (Chan and Decker, 1994). Under experimental conditions, Se-deficient animals should also be deficient in vitamin E, in order for white muscle disease to develop.

So far, the effect of vitamin E in skeletal muscle cell culture have never been studied. In our study, we assessed the effects of vitamin E on differentiation and cell detachment in cultured muscle cells. Vitamin E increased the rate of differentiation of Se-deficient muscle cells but had no effect on reducing loss of Se-deficient myotubes. These observations suggest that vitamin E can replace, to a certain extent, Se to maintain differentiation. This indicates that a part of selenium's role during differentiation is that of an anti-oxidant. On the other hand, vitamin E did not completely restore normal rates of muscle cell differentiation, this also suggests a possible role of Se as other than an anti-oxidant.

Another possible explanation for incapability of vitamin E to fully compensate for Se-deficiency is availability of vitamin E to the cells. In livestock, the effectiveness of vitamin E to provide protection against Se-deficiency disease has been shown to be varied, depending on its preparation (Whanger et al., 1976). Thus, maybe vitamin E used in this study was not available to the cells.

Cell Death

We are currently attempting to identify how Se-deficiency caused cell death. Identification of nature of cell death has received increased attention past decade (Hale et al., 1996). There are two different ways that cells can die: necrosis or apoptosis. Apoptosis is sometime referred to as "programed cell death", indicating that this process involves a precise series of molecular interactions inside the cell. A great number of apoptosis inducers have been identified so far, and oxidative stress has been shown to be

one of the causes of apoptosis in skeletal muscle cells (Sangel et al., 1996). Therefore, if muscle cells died by means of apoptosis during Se-deficiency, it is a good indication of involvement of oxidative activity in the cell death. However, our tests to show DNA fragmentation, one of the defining characteristics of apoptosis, has been unsuccessful.

Chapter 4. Conclusion

We found both advantages and disadvantages with application of serum-free medium for muscle cells. One of the advantages was that SF medium allowed precise control of the extracellular environment. For example, in this study, we obtained a low selenium status in the muscle cells. Another advantage in using this medium was its consistency in stimulating cell differentiation. L8 muscle cells are difficult to differentiate; however, we were very successful in inducing differentiation with SF media. SF medium usually stimulated differentiation more effectively than equine serum-supplemented medium.

Some limitations of serum-free medium were also noted during the course of this study. Obviously, SF medium lacks some of the nutrients and growth factors found in animal serum. SF medium did not support L8 cells as efficiently as equine serum. More "blebbing" (usually dead cells) were seen during differentiation using SF medium. Another limitation in using our SF medium may be that it eliminated some of the selenium-protein interactions which could be occurring with animal serum supplementation.

This study showed that two of the selenoproteins changed its status during differentiation due to different Se treatments. Glutathione peroxidase activity decreased over time under Se-deficient treatment. Selenoprotein W expression was initially undetectable and remained this way in Se-deficient media, but its expression was detected 24 hours after Se supplementation. Additional studies with selenoprotein W in L8 muscle cells may give more basic information on this protein, including its function and role in

skeletal muscle cells. Overall, this serum-free medium was useful by showing effects of low Se in L8 muscle cells.

Delaying skeletal muscle cell differentiation suggested that the Se-deficiency not only affects myotubes, but it also affects early skeletal muscle development. Increased L8 myotube degeneration (cell death) confirmed the loss of muscle cells due to Se deficiency as seen in WMD afflicted animals. Although the details of the biochemical interactions were not studied, these observations could explain a portion of the pathological morphology seen in white muscle disease. The convenience of repeating the study in cell culture system will readily give researchers the opportunity to investigate biochemical and molecular mechanisms of myopathy triggered by Se deficiency.

An interaction between vitamin E and Se was only significant during differentiation. Even during differentiation, vitamin E did not fully compensate for the effect caused by Se-deficiency. Vitamin E did not have any significant effect on L8 cell detachment. There are two possible explanations for this result: (1). the effect of Se deficiency may not be solely due to the loss of antioxidative selenoproteins, or (2). the vitamin E added to the media was not available to L8 muscle cells.

In conclusion, some suggested future studies include:

1. Development of serum free medium which would support proliferation of L8 skeletal muscle cells. This may allow cells to grow with low Se levels, thus obtaining a very low Se cell population.

2. Use of muscle cells from other animals, especially sheep. To date, ovine skeletal muscle cell lines are not commercially available. Obtaining satellite cells is the other option of obtaining ovine cell lines.

3. Investigation of status of other selenoproteins such as PHGSH and selenoprotein P during Se-depletion of the muscle cells. They may provide further important knowledge of the role of selenium in skeletal muscle cells.

4. A description of the mode of cell death. The observations of this study model may provide further understanding of the muscle atrophy in general as to whether this is due to apoptosis or to necrosis.

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