Polypeptide Factors Influencing Muscle Growth

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Introduction

The ability of producers to provide consumers with economically-priced lean meat is dependent upon both the regulation of muscle growth and the partitioning of nutrients to muscle tissue rather than to fat. Genetic selection, as well as improved animal nutrition and health have all been utilized to increase the rate and efficiency of muscle growth. However, muscle growth is also known to be greatly influenced by hormones and other factors found in blood serum. The identity of these factors and the mechanisms by which they regulate muscle growth are largely unknown. Their identification and isolation will set the stage for using recombinant DNA technology to produce large quantities of growth factors for use in animal production systems. In addition to its implications for animal agriculture, this research could have tremendous human impact in the areas of growth disorders, muscle wasting diseases, and muscle regeneration.

Circulating Peptide Growth Factors

To date, only a few serum-borne peptides affecting muscle accretion have been identified and in most cases their mechanism of action remains obscure.

Somatotropin

The effect of somatotropin deficiency on muscle growth has been well established for many years. Additionally, long-term administration of somatotropin to pituitary-intact animals has been reported to increase muscling, decrease fat content and improve feed efficiency in swine (Machlin, 1972; Chung et al., 1985); to increase nitrogen retention in steers (Mosely et al., 1982) and sheep (Davis et al., 1969); to increase growth rate in lambs (Wagner and Veehuizen, 1978); and to improve milk production in dairy cattle (Peel et al., 1981). However, there remains some question as to whether somatotropin has a direct effect on proliferation and protein turnover in muscle cells. Although there is an increased incorporation of ^{3}H-thymidine into DNA in muscle from somatotropin-treated hypophysectomized rats as compared to untreated controls (Breuer, 1969), these increases may reflect an effect of somatotropin on proliferation of nonmuscle cells or an indirect effect of somatotropin on muscle cell proliferation. It has also been reported that in in vitro incubations using rat diaphragm muscle, 10^{-8}M somatotropin stimulates amino acid uptake (Kostyo and Engel, 1960; Hjalmarson, 1968; Albertsson-Wikland and Isaksson, 1976). However, recent observations that many types of cells can secrete somatomedin (Hill et al., 1986; Adams et al., 1984; 1983), raise the possibility that responses seen in the intact diaphragm are the result of locally produced somatomedins. Consequently, it is generally believed that much if not all of the effect of somatotropin on muscle growth is mediated through somatotropin-dependent plasma factors – somatomedins – that are produced in response to somatotropin (Daughaday, 1981).

In culture, muscle cells do not appear to respond to addition of physiological levels of somatotropin. Florini and coworkers (Florini et al., 1977; Merrill et al., 1977; Ewton and Florini, 1980) have reported that somatotropin has no detectable effect on anabolic process in embryonic muscle cell cultures. Additionally, Allen et al., (1983) have reported that somatotropin has no direct effect on the rate of actin synthesis in myotube cultures derived from rat satellite cells. These findings support the theory that the effect of somatotropin on muscle is an indirect one mediated through the somatomedins. However, it should be pointed out that cultured muscle cells may be more analogous to embryonic cells than to muscle cells found in postnatal muscle tissue. Since somatotropin does not affect the embryo, it may not be surprising that cultured muscle cells are also unresponsive to somatotropin. Consequently, while the direct effect of somatotropin on muscle growth remains questionable, it cannot be unequivocally ruled out based on current data.

Insulin-Like Growth Factors (Somatomedins)

Insulin-like growth factors (IGF's) are small polypeptides (molecular weight approximately 7500) extracted and purified from human serum. These peptides possess insulin-like properties in vitro; however they do not cross-react with insulin antibodies (Van Wyk and Underwood, 1978; Zapf et al., 1978; Phillips and Vassilopoulou-Sellin, 1980). Multiplication stimulating activity is the name given to a family of polypeptides isolated from media conditioned by a Buffalo rat liver cell line (BRL 3A) (Moses et al., 1980). To date, two classes of insulin-like growth factors have been characterized. Insulin-like growth factor I (Rinderknecht and Humbel,

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1978), also referred to as basic somatomedin (pI 8.2-8.4) Bala and Bhaumick, 1979) or somatomedin C (Van Wyk et al., 1974; Svoboda et al., 1980); and insulin-like growth factor II (Rinderknecht and Humbel, 1978) or neutral somatomedin. Multiplication stimulating activity appears to be the rat form of IGF II, since the primary structure of MSA shows 93% identity with that of human insulin-like growth factor II (Marquardt et al., 1981). At concentrations of 10^{-9} to 10^{-10}M, IGFs are mitogenic for a variety of cultured cell types.

Biologically active receptors for both IGF I/SmC and IGF II/MSA have been identified on the surface of cultured muscle cells (Ballard et al., 1986; Bequinot et al., 1985; Yu and Czech, 1984). IGF I/SmC has been shown to stimulate growth of hypophosectomized rats (Schoenle et al., 1982), proliferation of cultured myoblasts (Ballard et al., 1986; Hill et al., 1986), amino acid uptake in cultured myoblasts (Hill et al., 1986), differentiation of cultured myoblasts (Schmid et al., 1983; Ewton and Florini, 1981), and RNA synthesis and polypeptide chain initiation in an isolated muscle (Monier and Le Marchand-Brustel, 1984). IGF II/MSA has been shown to stimulate proliferation of cultured myoblasts (Ballard et al., 1986; Hill et al., 1986; Florini et al., 1984; Florini and Ewton, 1981; Ewton and Florini, 1981; 1980), differentiation of cultured myoblasts (Florini et al., 1984; Florini and Ewton, 1981; Ewton and Florini, 1981; 1980), amino acid transport into cultured muscle cells (Janeczko and Etlinger, 1984; Merrill et al., 1978), and protein synthesis rate in cultured myotubes (Janeczko and Etlinger, 1984). Additionally, MSA has been shown to decrease protein degradation rate in cultured myotubes (Janeczko and Etlinger, 1984). In addition to their well-documented presence in serum, both IGF I/SmC and MSA have also been reported to be released by rat myoblasts (Hill et al., 1986; 1984), thus raising the possibilities that these peptides may be involved in autocrine or paracrine regulation of muscle growth. Based on the preceding evidence, it appears likely that insulin-like growth factors are potent stimulators of all aspects of muscle growth and development.

Insulin

The role of insulin in regulating general cell metabolism has been recognized for many years; however, the mechanism by which this regulation is accomplished is still not well understood. Similarly, the role of insulin in controlling muscle growth is not clear at the present time. Several lines of evidence suggest that insulin may have an anabolic effect on muscle tissue. Studies of a variety of animal models have demonstrated that wasting of skeletal muscle is a prominent feature of diabetes mellitus and that this wasting is reversed by administration of insulin to affected animals (Pain and Garlick, 1974; Flaim et al., 1980). Additionally, ribosomes isolated from muscle of diabetic rats are less active in in vitro protein synthesis systems than ribosomes from nondiabetic controls. In vitro studies with isolated muscles (Manchester, 1970, Fulks et al., 1975; Frayan and Maycock, 1979) and the perfused rat hemicorpus (Jefferson et al., 1977) have shown that insulin increases rate of protein synthesis and decreases rate of protein degradation in these systems.

In cultured muscle cells as well as fibroblasts and fibroblastic cell lines, supraphysiological concentrations of insulin, 1 μg/ml or higher, are required to elicit a maximal response. In muscle cell cultures, these high concentrations stimulate both proliferation and differentiation of myogenic cells (Merrill et al., 1977; Ball and Sanwall, 1980; Ewton and Florini, 1981). Additionally, insulin at high concentrations (10^{-6}M) is a component of synthetic medias used to support growth and differentiation of myogenic cells in culture (Florini and Roberts, 1979; Dollenmeier, 1981). It has been proposed that the stimulation of growth of fibroblasts by insulin is mediated by its weak binding to receptors for insulin-like growth factors (Rechler et al., 1976; 1977). Affinity-cross-linking studies have shown the existence of two classes of IGF receptors. Type I receptors (Chernausek et al., 1981; Massague and Czech, 1982; Kull et al., 1983) have a higher affinity for IGF I than for IGF II and have a low affinity for insulin. The structure and subunit composition of these IGF I receptors is very similar to that of the insulin receptor. Type II receptors bind IGF II with a higher affinity than IGF I and do not appear to have appreciable affinity for insulin (Massague and Czech, 1982; August et al., 1983; Perdue et al., 1983; Oppenheimer and Czech, 1983). At high concentrations, it is thought that insulin may bind to the type I receptor and in so doing affect cell growth in a manner similar to that observed for much lower concentrations of IGF I. This hypothesis is based on work by King et al., (1980) who showed that blockade of the high affinity insulin receptor with anti-receptor Fab fragments blocked high affinity insulin binding but did not prevent insulin-induced stimulation of DNA synthesis in cultured fibroblasts. Furthermore, these investigators showed that anti-insulin-receptor IgG, which triggers a number of acute insulin-like metabolic effects, did not stimulate DNA synthesis. These workers concluded that the growth-promoting effects of insulin on human fibroblast were due to binding of insulin to the Type I IGF receptor. Although this has not been proven to be the case in cultured muscle cells, it would seem quite likely that the well-documented effects of supraphysiological concentrations of insulin on proliferation and differentiation of cultured muscle cells are the result of this spillover action of insulin through IGF I receptors.

Insulin has a wide range of effects on cell metabolism. Consequently, it is possible that physiological levels of insulin may facilitate muscle cell growth by maintaining cells in a metabolic state that allows them to respond to other hormones and growth factors which stimulate cell proliferation.

Differentiation Inhibitor

Recently, Florini and coworkers (Florini et al., 1984; Evinger-Hodges et al., 1982) have reported that Coon's Buffalo rat liver (BRL) cells secrete a protein which is a potent inhibitor of skeletal myoblast differentiation in vitro. This peptide has an apparent molecular weight of approximately 30,000 to 36,000 and exhibits no detectable mitogenic activity. In skeletal myoblast cultures, it has been shown to reversibly block fusion, elevation of creatine kinase and increased binding of α-bungarotoxin. This inhibitor has also been isolated from sera of embryonic origin, and thus Florini and coworkers have suggested that it may play a role in embryonic growth of myoblasts and in satellite cell formation.

Transferrin

Transferrin is an iron-binding glycoprotein which is present in serum (Ozawa and Kohama, 1973) and embryo extract (li et al., 1981; 1982). Additionally, transferrin-like molecules have been isolated from both nerve and muscle extracts (Matsuda et al., 1984a; 1984b; li et al., 1982; Markelonis et al., 1982). In muscle cell cultures, iron-saturated transferrin stimulates both proliferation and differentiation and is essential for maintenance of healthy myotubes (Ozawa and Hagiwara, 1982). The effect of transferrin on muscle growth in culture is absolutely dependent on the presence of iron and appears to be class specific (i.e., mammalian transferrins do not affect avian myoblasts nor do avian transferrins affect mammalian myoblasts) (Shimo-Oka et al., 1986).

Fibroblast Growth Factor (FGF)

In cell cultures, FGF stimulates proliferation of myogenic cells and delays their differentiation (Gospodarowicz et al., 1976; Linkhart et al., 1981; Hauschka et al., 1985; Lathrop et al., 1985a; 1985b). Additionally, Allen et al., (1984) have recently proposed FGF as a regulator of satellite cell proliferation in skeletal muscle. However, it should be noted that these workers do not believe that serum is the source of FGF that is affecting satellite cell proliferation. Instead, they hypothesize that FGF-like molecules are produced locally in muscle and that these molecules are responsible for triggering a localized response of satellite cells during muscle regeneration.

In order to make progress in controlling animal growth, we need to better understand the mechanism by which the preceding factors regulate proliferation, differentiation and protein turnover in muscle cells. Additionally, discovery of factors such as the differentiation inhibitor described by Florini et al., (1984; 1982) suggests that there may be unknown serum factors which significantly influence the development of muscle tissue. Consequently, we need to increase efforts to identify and isolate these factors and to elucidate their mechanism of action. Recent advances in biotechnology provide the tools necessary to begin these studies aimed at understanding and regulating the mechanisms controlling accretion of muscle tissue in meat-producing animals.

Experimental Models For Studying Muscle Growth

In large measure, our inadequate understanding of the mechanisms controlling muscle growth in meat animals is the result of difficulties encountered in devising a satisfactory model system in which to study these processes. Experimental animals, isolated muscles and muscle cell culture have been the primary systems used to study the effects of specific peptides on the growth of muscle tissue.

While experimental animals provide the most biologically complete system in which to study muscle growth, the complex interactions of their hormonal systems and large animal-to-animal variation often make it difficult to evaluate the role of any specific factor in muscle growth. Additionally, experiments with animals are expensive, labor intensive, and often require several weeks or months to complete. In order to evaluate the effect of a specific factor on muscle growth, it is also necessary to measure the muscle mass of control and experimental animals. At present, techniques available for measuring muscle mass are laborious and inaccurate.

In vitro incubation of excised muscle tissue has also been used to study the effects of various peptides on muscle growth. This system has been used primarily to study the effects of various substances on the rates of protein synthesis and degradation in skeletal muscle tissue (Fulks et al., 1975). This technique provides a more controlled experimental environment and easier measurement of protein synthesis and degradation rates than does the whole animal. However, a major concern in this system is the fact that excised muscles are generally in a catabolic state relative to protein turnover (e.g., protein degradation exceeds protein synthesis) (Fulks et al., 1975; Clark and Mitch, 1983; Goldspink et al., 1983).

Muscle cell culture has also been used extensively to study the effects of specific peptides on both protein turnover and muscle cell proliferation. In culture, muscle precursor cells differentiate and proliferate to form myoblasts which fuse to form multinucleated myotubes. Myotubes synthesize contractile proteins, assemble them into myofibrils and develop the ability to contract. However, in order for these processes to occur, the culture media must contain blood serum or serum factors. Presumably, serum contains specific factors that are necessary for the differentiation and proliferation of muscle cells in culture. Consequently, muscle cell culture has been used extensively to study the effect of specific factors on the proliferation, protein turnover and differentiation in muscle cells. Although cell culture lends itself well to these kinds of studies, there is some concern about whether cell culture findings are valid for muscle tissue in vivo. Therefore, cell culture data must ultimately be confirmed in the animal as a final test of its validity.

Nonetheless, we believe that muscle cell culture provides a useful tool for identifying factors which influence muscle growth and for determining their mode of action in meat animals. Consequently, utilizing the L6 muscle cell line isolated by Yaffe (1969), we have developed muscle cell culture bioassays for measuring the effects of porcine sera and serum fractions on proliferation and protein turnover in cultured muscle cells. In these assays, porcine serum stimulates proliferation and protein synthesis and inhibits protein degradation in a concentration-dependent manner, thus establishing that the L6 myogenic cells do indeed respond to factors present in swine sera. Therefore, we believe that the bioassays we have developed can be used in conjunction with biochemical and immunological techniques to isolate these factors and to elucidate their mode of action on muscle cells.

In order to establish the validity of the muscle cell proliferation and protein turnover bioassays, we have used them to evaluate sera obtained from pigs before and after imposition of treatments that have been reported to change the rate and/or efficiency of muscle growth in swine. In these initial studies, our goal was to determine if the bioactivity of sera obtained before and after a particular treatment reflected these changes. Sera obtained before and after porcine

somatotropin injection, fasting, or feeding of subtherapeutic levels of antibiotic were examined.

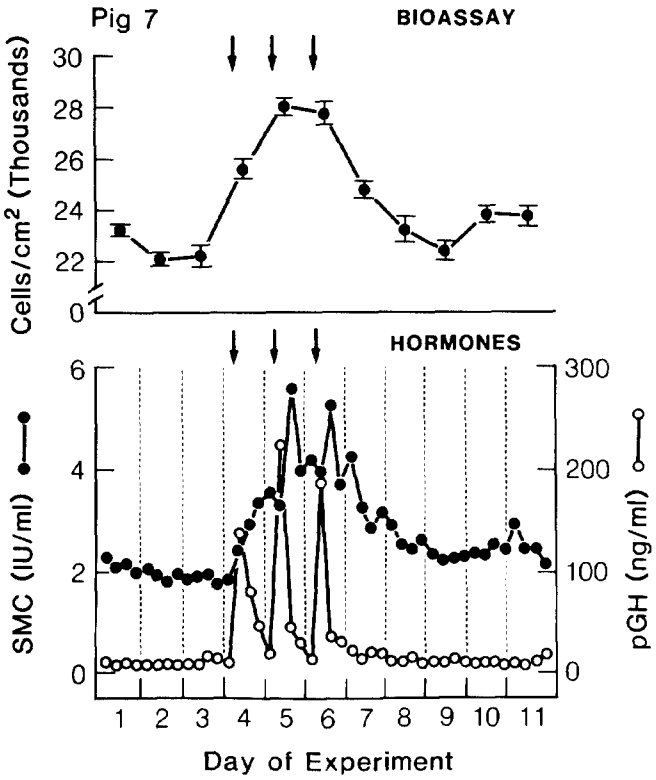
Effect of Sera Obtained from Pigs Before and After I.M. Porcine Somatotropin Injections on Proliferation and Protein Turnover in Cultured Muscle Cells

The ability to utilize genetic engineering to produce large quantities of somatotropin or to insert it into the genome of domestic animals has focused considerable attention on the anabolic effects of this peptide in meat-producing animals. Although there have been conflicting reports concerning the effect of exogenous somatotropin on muscle growth in swine with normal endogenous somatotropin levels, it now appears that long-term injection of highly purified porcine somatotropin increases muscling, decreases fat and improves feed efficiency in growing pigs (Chung et al., 1985). The potential use of exogenous somatotropin as a growth promotant is hampered by the fact that its mode of action on muscle and adipose tissues is still poorly understood. Indeed, studies using cultured muscle cells have shown that somatotropin exerts little, if any, direct effect on various indices of muscle growth. However, Florini et al., (1980) have shown that administration of somatotropin to hypophosectomized rats increases the ability of their sera to stimulate proliferation of cultured myogenic cells. This finding suggests that, at least in hypophosectomized rats, restoring somatotropin levels to near normal may stimulate the release of mitogenic substances (e.g., somatomedins) into the blood.

In order to determine whether injection of porcine somatotropin into normal growing pigs alters the levels and/or activity of factors affecting muscle growth, we have examined the ability of porcine sera obtained before and after somatotropin injection to regulate proliferation and protein turnover in cultured muscle cells. In this study eight 19-kg barrows were injected intramuscularly with porcine somatotropin once daily for three days. Three barrows received 0.027 IU/kg/day (low dose) and five received 0.21 IU/kg/day (high dose). All animals were catheterized and allowed to recover for two days prior to the start of the experiment. Blood samples were collected from each pig every six hours for the duration of the experiment (12 days). Pre-injection blood samples were collected from each pig for three days prior to injection with porcine somatotropin. During this period, each pig was injected once a day with sterile saline in the same manner later used to inject somatotropin. On days 4 to 6, each pig received the appropriate amount of somatotropin in a daily intramuscular injection. Serum samples were combined to give 24-hr pools for each pig and these samples were assayed in the proliferation assay. Individual 6-hr serum samples were assayed for somatotropin, SmC, insulin and cortisol.

Although injection of the low dose of somatotropin significantly increased somatotropin level in the serum, it did not significantly increase mitogenic activity or SmC levels in the serum. In contrast, injection of the high dose of somatotropin significantly ($P<.005$) increased the mitogenic activity of the serum as well as the circulating levels of somatotropin and SmC (Fig. 1). It should be noted that SmC concentration and mitogenic activity varied independently in some instances,

Figure 1



Proliferation-promoting activity, SmC and pGH levels in sera from pigs injected with .143 mg pGH/kg/day. Injections (indicated by arrows) were given at 2 p.m. on days 4, 5 and 6. In the bioassay, triplicate cultures were assayed in the presence of media containing 3.5% of the 24-hr serum pool obtained on the indicated day. Individual 6-hr points pooled to form 24-hr serum pools are indicated by the vertical dotted lines. RIAs were performed on sera obtained from each 6-hr blood sample.

suggesting that factors in addition to SmC may affect the mitogenic activity of the sera. This is dramatically illustrated by the fact that serum from one of the high-dose pigs did not show increased mitogenic activity, even though SmC levels in the serum were elevated after somatotropin injection.

Our studies have also shown that sera obtained from pigs before and after porcine somatotropin injection have different effects on protein turnover in cultured muscle cells. Sera obtained after somatotropin injection stimulates muscle protein synthesis and decreases muscle protein degradation as compared to sera obtained prior to injection (Tables 1 and 2). These findings support the theory that somatotropin injections increase the levels and/or activities of serum factors which are involved in hypertrophic growth of muscle in culture.

Effect of Swine Sera Obtained Before and After Feeding of Subtherapeutic Levels of Antibiotic on Proliferation and Protein Turnover in Cultured Muscle Cells

Although antimicrobial compounds, at subtherapeutic levels, are used extensively by swine producers as growth promotants (Jukes, 1972; Thrasher et al., 1979; Yen et al.,

Table 1. Effect of Sera Obtained Before and After pGH Injection, Supplementation with Subtherapeutic Levels of Antimicrobials, or Fasting on Protein Synthesis in L^6 Myotubes

	Synthesis[a] *(%)*	*Significance*	*Difference*[b] *(%)*
Pre-pGH	18.3	P<.01	+36.1
Post-pGH	24.9		
Pre-Asp250	25.6	P<.5	NS
Post-Asp250	29.1		
Pre-fast	22.2	P<.5	NS
Fasted	18.5		

[a]Synthesis for each serum treatment is expressed as a percentage increase or decrease relative to control cultures containing serum free media.

[b]Percentage increase or decrease in synthesis in cultures treated with post sera as compared to cultures treated with the corresponding pre sera.

Table 2. Effect of Sera Obtained Before and After pGH Injection, Supplementation with Subtherapeutic Levels of Antimicrobials, or Fasting on Protein Synthesis in L^6 Myotubes

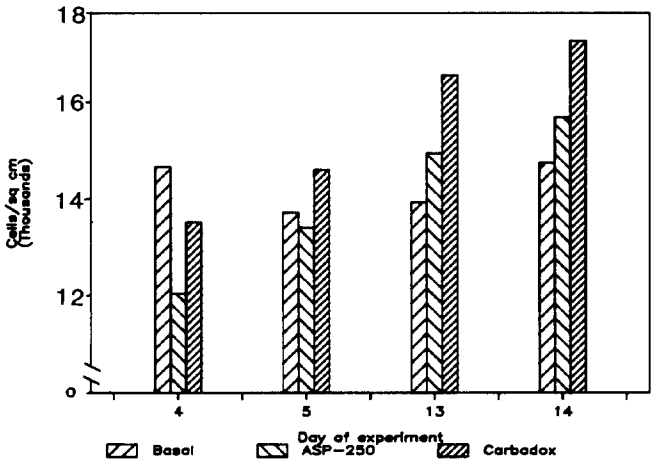
	Degradation[a] *(%)*	*Significance*	*Difference*[b] *(%)*
Pre-pGH	−15.4	P<.01	−32.5
Post-pGH	−20.4		
Pre-Asp250	−19.1	P<.025	−23.6
Post-Asp250	−23.6		
Pre-fast	−16.2	P<.05	+26
Fasted	−12.7		

[a]Degradation for each serum treatment is expressed as a percentage increase or decrease relative to control cultures containing serum free media.

[b]Percentage increase or decrease in degradation in cultures treated with post sera as compared to cultures treated with the corresponding pre sera.

1976; Moser et al., 1980; Veum et al., 1980), the physiological mechanism by which antimicrobials stimulate weight gain and improve feed efficiency has not been unequivocally established. Several mechanisms have been proposed to explain the stimulatory effect of antibiotics on growth; however, no physiological or chemical change that is clearly responsible for increased growth rate has been observed in supplemented pigs. However, we now have evidence that feeding subtherapeutic levels of antibiotic to growing pigs increases the ability of their sera to stimulate proliferation and decrease protein degradation in cultured myogenic cells. Additionally, we have shown that these effects are not a direct action of the antibiotic but, rather, are the result of an antibiotic-induced change in the serum.

Figure 2



Mitogenic activity of DMEM plus 2% serum obtained from pigs before and after antimicrobial supplementation. Pooled data from 3 pigs/carbadox diet, 4 pigs/asp250 diet and 3 pigs/basal diet are shown. Mitogenic activity is expressed as number of cells/cm^2. On days 4 and 5 all pigs received basal control diets. After the last blood collection on day 5, pigs received the indicated treatment diet and blood was again collected on days 13 and 14. Carbadox and Asp250 stimulated proliferation above basal control days (P<.001 and P<.005, respectively). There was no significant change in mitogenic activity of sera from pigs maintained on the basal diet throughout the study.

Figure 2 shows the proliferation promoting activity of sera from pigs before and after feeding of either ASP-250 or carbadox. For comparison, the proliferation promoting activity of serum from a pig maintained on a basal diet (no antibiotic) throughout the experiment is also shown. Antibiotic supplementation for only 5 days results in a substantial increase in the mitogenic activity of the sera. Three separate studies each containing 8 pigs have shown that either ASP-250 or carbadox supplementation significantly (P<.005) increases the mitogenic activity of serum in the muscle cell culture bioassay. However, addition of carbadox to diets already containing ASP-250 results in no increase in serum proliferation promoting activity above that already observed as a result of the ASP-250 supplementation. These findings suggest that the mechanism of action of ASP-250 and carbadox is similar if not identical. As compared to the pre-antibiotic sera, post-antibiotic sera causes no change in rate of total protein synthesis while decreasing rate of total protein degradation (Tables 1 and 2). Addition of either ASP-250 or carbadox to the cell culture media or to sera obtained from control pigs has no effect on the proliferation rate of the muscle cells; therefore, the antibiotic-induced changes in serum proliferation promoting activity are not caused by the direct affect of the antibiotics on the muscle cells. Based on these preliminary studies, it appears that the muscle cell culture bioassays may be useful in identifying growth factors whose levels and/or activities change in response to antibiotic supplementation.

Effect of Sera Obtained from Pigs Before and After Fasting on Proliferation and Protein Turnover in the Muscle Cell Culture Bioassays

Because nutrient deprivation causes rapid and dramatic changes in muscle growth, fasting has long been used as a

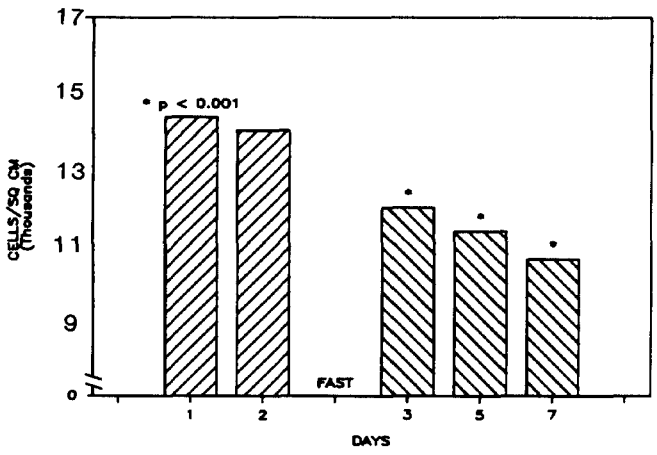
model for studying the metabolic and hormonal factors that regulate growth of muscle tissue. Fasting has been reported to increase the rate of muscle protein degradation (Li and Goldberg, 1976; Goodman et al., 1981; Dunn et al., 1982; Lowell et al., 1986; Dahlmann et al., 1986) and to decrease the rate of muscle protein synthesis (Li et al., 1979; Bates and Millward, 1983, Garlick et al., 1983; Stirewalt, 1984). Additionally, maternal nutrient deprivation has been reported to decrease postnatal satellite cell proliferation in offspring (Beermann et al., 1983). These effects of fasting on muscle may be mediated, at least in part, by changes in the level and/or activity of circulating factors, such as the somatomedins, which may regulate muscle growth. This hypothesis is supported by reports that fasting reduces the level and activity of somatomedins in sera (Merimee et al., 1982) and increases the serum level of factors that inhibit metabolism and sulfate uptake in in vitro costal cartilage bioassays (Salmon et al., 1983; Phillips et al., 1985). However, very little is known about the effects of fasting on serum factors that may affect muscle growth in an economically important species such as swine. Consequently, we have examined the effect of fasting on the ability of porcine sera to regulate proliferation and protein turnover in cultured muscle cells.

Barrows weighing 19 to 24 kg were fitted with jugular catheters and given a 48-hr recovery period. 12 ml blood samples were withdrawn from the catheters at 4 evenly spaced intervals between 8 am and 6 pm on days 1, 2, 3, 5 and 7 of the study. Pre-fast blood samples were taken on days 1 and 2 prior to the imposition of a 5-day fast at 8 pm on day 2. Fasted blood samples were taken on days 3, 5 and 7 of the study. Blood was allowed to clot and the 4 serum samples collected from an individual pig on a given day were pooled for testing in the muscle cell proliferation bioassays. Pre-fast or fasted serum pools formed by combining sera obtained on days 1 and 2 (pre-fast) or days 3, 5 and 7 (fasted) of the study were used in the protein turnover bioassays.

Figure 3 shows the pooled results of a study in which the muscle cell proliferation promoting activity of sera from 4 control and 4 fasted pigs was measured. The mitogenic activity of the sera from the unfasted control pigs was not significantly different on the pre-fast days as compared to the fasted days ($P<.5$). However, in all of the fasted pigs, a significant decrease ($P<.001$) in serum mitogenic activity was observed during the fasted days as compared to pre-fast days. The mitogenic activity of the sera was already decreased on day 1 of the fast (day 3 of the study) and remained depressed throughout the duration of the study. This relatively rapid drop in serum mitogenic activity upon initiation of the fast suggests that this reduction is the result of alterations in the levels or activities of highly regulated serum factors and not the result of long-term nutrient depletion. Mitogenic activity returned to near pre-fast levels by the fifth day of refeeding (data not shown). As expected, fasting also affects the ability of porcine sera to regulate protein turnover in cultured myotubes. Tables 1 and 2 show that while fasting does not alter the effect of sera on protein synthesis ($P<.05$), sera from fasted pigs increases protein degradation by 21.6% ($P<.05$) as compared to sera from pre-fast pigs.

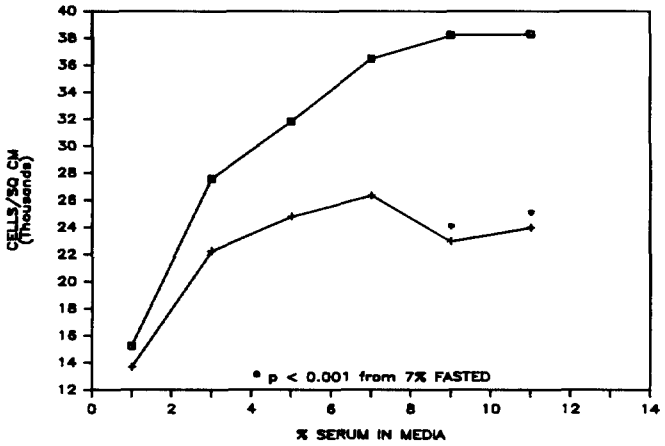
Studies in which humans have been fasted have shown that fasting causes a significant reduction in the level of somatomedin C (SmC) in the sera. Sera from fasted pigs show a similar reduction in SmC levels and this reduction may be partially responsible for the reduced proliferation and increased protein degradation observed in muscle cells treated with fasted sera as compared to those treated with pre-fast sera. However, we have data suggesting that factors other than SmC may also be involved in these responses. A dose-response curve measuring the effect of pre-fast sera on proliferation (Figure 4) shows that mitogenic activity increases with increasing serum concentration up to 7% where a plateau is reached. A similar curve for fasted sera shows a slower increase in mitogenic activity with concentration of sera up to approximately 4% serum. Media containing more than 7% fasted sera significantly inhibits the proliferation of

Figure 3



Mitogenic activity of DMEM plus 2% serum obtained from pigs before and during fasting. Pooled data from four, 20 kg pigs are shown. Activity is expressed as number of cells/cm^2. Days 1 and 2 are pre-fast days, and days 3, 5, and 7 are fasted days. Asterisk (*), indicates statistical significance ($P<.001$) from the 2 pre-fast days.

Figure 4



Effect of increasing concentrations of pre-fast (----) and fasted (+----+) serum pools on proliferation of cultured myogenic cells. Mitogenic activity is expressed as the number of cells/cm^2 (n=3 for each treatment). Serum concentration in the culture media is expressed as % (v/v). Asterisk (*), indicates a significant difference ($P<.001$) from the mitogenic activity of 7% fasted serum.

muscle cells as compared to media containing 4% to 7% sera ($P<.001$). This result suggests that, in addition to decreasing the level and/or activity of serum mitogens such as SmC, fasting may also increase the level of serum factors which inhibit proliferation of muscle cells in culture.

Summary

We have shown that fasting, i.m. porcine somatotropin injection and feeding subtherapeutic levels of antibiotic alter the ability of porcine serum to regulate proliferation and protein turnover in cultured muscle cells. Sera obtained from pigs before and after each of these treatments provides a starting point for fractionation studies aimed at identifying serum bone factors which influence muscle growth in meat animals. These studies will utilize conventional chromatography, HPLC and immunoaffinity chromatography to identify fractions which stimulate or inhibit proliferation and/or protein turnover in the muscle cell culture bioassays. These studies should provide added insight into the mode of action of known growth factors and may possibly lead to identification of unknown factors which regulate muscle growth. However, because control of muscle growth is a complex biological process involving growth factors, cell surface receptors and cellular responses, the task of identifying factors involved in this process and defining their mode or action will be long and arduous.

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Discussion

D. Campion: Bill, you have talked about the possibility of identifying new mitogenic factors and also inhibitory factors. Have you taken your work far enough to know at least what molecular weight range you are working in?

W. Dayton: Yes, we know the molecular weight range of the inhibitor is probably something less than 50,000. We know that mitogenic compounds are found both in the 150,000 molecular weight range, which is where the IGF certainly would be found, and also in the lower molecular weight range below 60,000. The question there is whether or not this is an IGF bound to a carrier protein of some sort that is biologically active or whether it is in fact a different or unknown growth factor. I think that whether or not it turns out to be a new growth factor, it should give us some insight into the mode of action of existing growth factors, if in fact that is what we end up with.

Campion: I guess I would ask another question then, in terms of the inhibitor, whether that is an inhibitor of somatomedin activity. Have you looked for competitive versus noncompetitive inhibition with the insulin-like growth factors?

Dayton: We haven't done that yet and we don't know; but that's a very reasonable question and we need to do that as we get more purified compound to work with.

D. Devol: Did you say whether or not you are using a radioimmunoassay or a bioassay for somatomedin?

Dayton: We're using a RIA for the somatomedins at this point, the actual numbers I gave you.

Question: You mentioned that in some instances at post-injection you did not see a change in somatomedin level but you did see a cell response – a proliferation response. Would you expect possibly different results if you used a bioassay instead of RIA?

Dayton: Twofold. First of all, I didn't really say. But what we normally saw was a change in the RIA levels and sometimes not in the cells. Yes, I think so. Basically, what we are seeing here, what we are using here, I believe, is that the culture system is probably very comparable to what you would get from a bioassay. The RIA, of course, is different, complicated possibly by either the recognition of non-active somatomedin or by possibly the carrier proteins and their interference in the RIA's. These were all acid-extracted and we did everything we could to control that; but the RIA's are certainly not infallible when it comes to IGF's. That's one reason that we think the cell culture system may provide a useful adjunct to the RIA's because of the fact that the RIA's do not measure biological activity and in that regard can be misleading sometimes.

Question: Bill, finding growth factors is a very exciting business. Could you speculate about what you might hope to find beyond somatomedin C as a growth stimulator?

Dayton: I really can't. I don't know what we may see. I think that one of the areas, (I'm not sure we would find it in this particular approach – the way we are doing it now) – an area that is potentially very exciting is the *oncogene* product area. I don't know that the particular method we are using is the way to find that but I think there certainly is potential in that area where one may find some very exciting growth-related things there.

Question: Have you looked at any other growth factors, such as fibroblast growth factor or anything like this, or done anything with these types of compounds?

Dayton: Yes, we know that they are effective at least – others have looked at fiberblast growth factor, transferrin and most of the ones that I've indicated up here. The common ones.

M. Dikeman: Bill, the title of your presentation was "Factors affecting muscle growth" ... and yet you really didn't refer to some of the hormones, estrogen, testosterone. I was wondering what I am to interpret from their absence in your presentation?

Dayton: Well, personally, it was just a matter of time. Also, I think most of those have been covered before. The other thing that I personally believe about these particular kinds of compounds is that while they very well may have an effect on muscle growth, and I'm sure that is well established; the question of whether or not regulatory agencies will allow us to use them in a practical setting in terms of animal agriculture as growth stimulants, I think, is in essence fairly well decided. And that is that they probably won't. So from the standpoint of a practical kind of usable growth promotant, I would suspect that as a stimulant (something that is added exogenously), that some of these kinds of hormones are probably not very viable candidates. Not because they may not be effective but just because they are very unlikely to be approved. So it was a combination of time, having had it covered before, and what I just said about the potential approval problems. Not to mention that I don't think they work.

M. Hunt: Bill, this deviates slightly from the title of your

presentation but could you give us the current thinking on why muscle cells remove themselves from the cell cycle and how all that happens?

Dayton: I can give you the current thinking. I'm not sure I can give you a good answer. There really are a number of things probably involved in that. Certainly, the genetic programming of the cell, in the sense that there is some evidence that at some point during the differentiation of the cell, the cell cycle slows down dramatically and the cells simply develop the ability to fuse and may in fact be removed from the cell cycle by the fusion process, is one angle of that. The other theory, or actually it may be an adjunct theory, is that there are, in fact, circulating molecules such as the differentiation inhibitor, fibroblast growth factor, somatomedins and whatever which act upon the cells and cause them to do specific things at specific times in terms of their differentiation. The differentiation inhibitor, for example: If it does in fact inhibit the differentiation of the cells (which is to say it basically inhibits the fusion of myoblasts) may be a good candidate for something that may be at higher levels in early embryonic development and retard the fusion of the muscle cells; allow them to continue to proliferate, whereas levels may drop off in later embryonic life and allow the fusion process or the differentiation process to continue and lets the cells withdraw from the cell cycle. But I don't think we really understand enough; we know bits and pieces of what might control it but in terms of an integrated package of how all this is controlled, I don't think we know the answer yet. It is pretty clear that it is an interaction of the environment with the genetic differentiation program of the cell and triggering of particular genetic events. Not a very good answer, but I don't think we have a very good answer at this point.