Crushed Muscle Extracts: a Model System to Investigate Growth Factor Regulation of Satellite Cell Activities in Meat Animals

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Abstract

The manipulation of postnatal muscle growth has allowed agricultural producers to increase the yield of lean tissue from meat animals. Of importance to this growth process are the activities of postnatal muscle precursor cells, referred to as satellite cells. The proliferation and differentiation of satellite cells in muscle serves to regulate muscle growth by adding new DNA to the existing muscle fibers. *In vitro* data indicate satellite cell activities are controlled by numerous growth factors, each with different actions on different satellite cell activities. Recently, the use of extracts from damaged muscles has been an effective tool to analyze what specific growth factors may be important in the postnatal growth of muscle. This review explores the possible components of muscle extracts and relates the importance of these findings to the mechanisms regulating muscle growth, particularly the activation, proliferation, and fusion of satellite cells, as well as the maintenance of a satellite cell reserve.

Key words: muscle, satellite cells, growth, growth factors, myoblasts.

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The growth of muscle tissue is the ultimate goal of meat animal agriculture [5]. Most of the growth in muscle mass occurs in the early postnatal period which, conveniently, is a period amenable to pharmacologic and dietary manipulation by agricultural producers.

Muscle tissue can grow by *hypertrophy* (enlargement or increase of mass of muscle fibers), by *hyperplasia* (increase in numbers of fibers or in numbers of muscle nuclei), or by a combination of these two processes. Since nuclei in muscle fibers are incapable of DNA synthesis or mitotic division, increases in muscle fiber number or in numbers of muscle nuclei are due to proliferation and subsequent differentiation of skeletal muscle precursor cells known as *myoblasts* [5, 83, 88]. In certain situations, hypertrophy of adult skeletal muscle fibers can occur independently of myoblast proliferation [1, 34, 57]. However, early postnatal muscle fiber hypertrophy appears to be dependent upon the proliferation of postnatal myoblasts known as *satellite cells*, since skeletal muscle mass and muscle fiber DNA content are highly correlated [5, 63, 81], and normal postnatal growth of muscle tissue is reduced if the muscle is irradiated to prevent myoblast proliferation [71, 72, 73].

Satellite cells

Located between the basal lamina and muscle fiber membrane, satellite cells can be defined as the myoblast population contributing to postnatal growth and regeneration of skeletal muscle. Satellite cells have received detailed consideration in the context of adult muscle regeneration following injuries such as disease, trauma, ischemia or exhaustive exercise [17, 19, 76]. In these situations satellite cells located on the damaged fibers undergo a series of fundamental processes including activation from mitotic quiescence, proliferation, differentiation, and self-renewal. Following a muscle tissue injury, satellite cells on the injured muscle are "activated" from a G_0 state to enter the G_1 phase of the cell cycle. Once in the cell cycle, expansion of the satellite cell population occurs as these cells undergo proliferation. Ultimately, some of these daughter cells differentiate, and fuse into existing myofibers or form new fibers [19].

In addition to being able to generate nuclei for the repair of damaged or diseased muscle fibers, satellite cells are also critical in normal postnatal muscle growth. Although muscle fiber number does not increase appreciably after birth, the proliferation and subsequent fusion of satellite

cells in muscle serve to regulate muscle growth by adding new nuclei to the existing muscle fibers [5, 76, 88]. Autoradiographic studies using ^{3}H-thymidine-labeled satellite cells revealed that satellite cell fusion into adjacent myofibers accounts for the increased DNA content of postnatal muscle [63]. Estimates of muscle nuclei contributed postnatally in mammals range from 50-63% in pigs [42] to 73-88% in rats [27, 89]. Two studies have indicated that almost all (94-99%) muscle fiber DNA in birds is accumulated postnatally [62, 64]. A number of studies have indicated that muscle protein synthetic ability is correlated with muscle fiber DNA content, suggesting that increases in muscle mass are dependent upon satellite cell contributions [5]. Recent studies have shown that both compensatory muscle hypertrophy and hypertrophy associated with normal growth are diminished in muscles subjected to gamma irradiation in which satellite cell reproduction is eliminated [71, 72, 73]. These results confirm that satellite cell proliferation plays a role in hypertrophy of mature muscle.

Prior to growth cessation, the increase in myofiber DNA levels off and satellite cells become mitotically quiescent to function as a reserve stem cell population [88]. Mozdziak et al. [65] showed that there is an age-related decrease in turkey skeletal muscle satellite cell mitotic activity and an age-related increase in muscle cytoplasmic volume-to-nucleus ratio from 3 to 26 weeks of age. This study demonstrated that the turkey pectoralis thoracicus and biceps femoris undergo a significant late phase of growth without appreciable production of myonuclei by satellite cell proliferation. At this point, the activation of quiescent muscle satellite cells may become a critical step when considering means to increase lean muscle mass in production animals.

A large body of *in vitro* data indicate myoblast activities are controlled by a battery of growth factors, each with different actions on different myoblast activities. Since postnatal growth of muscle tissue appears to be dependent upon satellite cells, the effects of growth factors on each of the satellite cell activities (activation, proliferation, differentiation and maintenance of a stem cell reserve) are of special interest in regard to prospects for controlling postnatal muscle growth in agricultural animals.

Muscle extracts

Numerous investigators have recently shown that saline extracts produced by incubation of gently crushed or stretched adult muscles are bioactive in a variety of myoblast culture systems, including satellite cells [13, 14, 15, 16, 20, 21, 44, 51, 53, 54, 84]. Presumably, factors present in such extracts represent factors released from cells or extracellular matrices (ECM) following muscle injury. Growth factors present in the muscle extracts have been only partially characterized at this point. However, the use of extracts from damaged muscles (versus the more heterogeneous muscle homogenates), has been an effective means to analyze the specific growth factors which may be important in controlling the distinct satellite cell activities. Thus, the objectives of this review are to highlight the various studies using crushed or stretched muscle extracts, to explore the possible components of the extracts, and to relate the importance of these findings to mechanisms regulating muscle growth.

Activation

The initial step in muscle regeneration is the activation of muscle satellite cells from the quiescent G_0 state to the G_1 phase of the cell cycle. Bischoff [13] found that mitotically quiescent satellite cells attached to single fibers of adult rat muscle were induced to enter the cell growth-division cycle by addition of a saline extract of crushed rat muscle. Satellite cells exposed to rat crushed muscle extract (CME) entered the DNA synthesis (S) phase within 18 hours and proliferated with a generation time of 12 hours. However, the extract did not enhance proliferation of mature muscle-derived fibroblasts. Furthermore, little such activity was contained in crushed extracts from heterologous tissues such as liver, lung, heart and kidney [14] or in extracts from uncrushed muscle [21, 44].

The *in vitro* system refined by Bischoff [13, 14] is tedious and may not be suitable for generating large numbers of cells for biochemical analyses. Johnson and Allen [51] developed another technique for studying satellite cell activation. Their method measures proliferating cell nuclear antigen (PCNA) as a marker for cells entering the S phase of the cell cycle. Use of this technique has shown that satellite cells taken from young (3 wk) and mature (9 mos) rats express PCNA prior to increases in cell numbers after a lag period following isolation from the source tissue. The lag period for cells isolated from mature rats was longer than for cells isolated from younger rats, suggesting that cells from the younger rats were already in the cell cycle whereas cells from mature rats needed additional time to transit from G_0 to G_1. Addition of the basic form of fibroblast growth factor (FGF2) accelerated PCNA expression in cells isolated from younger rats, but FGF2 was much less effective on cells obtained from the older rats [51]. In contrast, addition of rat CME to the cultures accelerated PCNA expression in satellite cells from both age groups [51]. These observations and those of Bischoff's group indicated that CME contains a factor which may activate quiescent satellite cells to enter the cell cycle, and that FGF2 acts to stimulate satellite cell proliferation only after the activation event. Furthermore, specific binding of FGF can be detected at 18 h postplating on the cells isolated from young rats, but is not detectable until 42 h on satellite cells from old rats [52]. This observation suggests that activated, but not quiescent, satellite cells express FGF2 receptors. Although FGF2 and platelet-derived growth factor (PDGF) are activation factors for quiescent fibroblasts [38], they did not activate satellite cells in several studies [13, 14, 51, 52].

In contrast to these findings, Yablonka-Reuveni and Rivera [91] utilized a rat single-fiber system similar to Bischoff's and reported that incubation with FGF2 doubled the number of PCNA-positive satellite cell nuclei detectable on isolated, cultured muscle fibers. It is unclear

in these experiments whether the FGF2 functioned to activate satellite cells or to stimulate proliferation of previously activated cells. The age of the rats (3 mos, versus 9 mos in studies by Johnson and Allen) from which these cells were isolated may have contributed to the contrasting results. Since the kinetics of appearance of PCNA-positive cells were identical to those of MyoD-positive cells (a marker of satellite cell activation) [39, 80], this study seems to support the idea that FGF2 was functioning as an activation factor. The explanation for the conflicting results of this study with those of Bischoff [13] and Johnson and Allen [51, 52] concerning the role of FGF2 in satellite cell activation awaits further investigation.

Recently, Jennische et al. [47] found that expression of a heparin-binding growth factor, hepatocyte growth factor (HGF or scatter factor) was increased in regenerating skeletal muscle and also in growing postnatal muscle. Subsequently, Allen et al. [6] showed that HGF was able to activate quiescent rat satellite cells in the mass culture system described previously [51]. In addition, this group reported the presence of the protooncogene *c-met* (whose product is the signalling receptor for HGF) throughout the first 72 hours of satellite cell culture. Therefore, HGF is a prime candidate for the satellite cell activation factor in CME as evidenced by its presence in muscle, its induction upon muscle injury, the expression of its receptor by quiescent satellite cells, and its ability to activate quiescent satellite cells in a model system. However, it is still unknown whether only one activation factor is present in CME.

Proliferation

In addition to being able to activate quiescent satellite cells, CME has been shown to be effective in stimulating proliferation of activated satellite cells. Extracts obtained from crushed rat muscles stimulated the proliferation of myogenic cells from rat embryos (assessed by ^{3}H-thymidine [^{3}H-TdR] incorporation into DNA) and stimulated muscle growth *in vivo* (as measured by an increase in DNA content) after intramuscular injection into rat pups [14]. Increasing concentrations of murine CME caused a dose-dependent increase in ^{3}H-TdR incorporation into DNA by primary mouse myoblasts [21] and C2 myoblasts, a mouse myoblast cell line generally considered to represent a satellite cell line [21, 44]. Haugk et al. [44] demonstrated that increasing concentrations of bovine CME caused a dose-dependent increase in ^{3}H-TdR incorporation by C2 myoblast cultures, while corresponding extracts from uncrushed muscle did not. Furthermore, Haugk et al. [44] demonstrated that both murine and bovine CMEs contained approximately twice as much total protein as extracts from uncrushed muscles, suggesting that damage to muscle cell membranes or to ECM releases numerous proteins among which are probably the growth-promoting compounds.

Summers et al. [84] explored the involvement of soluble growth-promoting factors in stretch-induced hypertrophy of chicken patagialis muscle, showing that stretched-muscle extracts produced a dose-dependent stimulation of embryonic chick myoblast proliferation *in vitro*. Passive stretch for 5 d produced a rapid hypertrophy of the patagialis muscle which was accompanied by a dramatic increase in the mitogenic activity of the extracts. Release of the stretch resulted in an arrest of muscle growth and an immediate decline in mitogenic activity. Similarly, low-salt extracts from chicken anterior latissimus dorsi muscles have been shown to exert a pronounced mitogenic effect on cultured chick myoblasts and rat L6 myoblasts [53]. This was evidenced by a substantial increase in total cellular protein, and increases in DNA synthesis. The stimulation of L6 myoblasts indicates that, as in the bovine extract prepared by Haugk et al. [44], the mitogenic activity of the chicken extract does not appear to be species-specific.

These studies indicate that crushed or stretched muscle extracts contain potent muscle cell mitogens that are active across species. It is likely that these mitogens are a combination of growth factors. Possible mitogenic components of CME and their roles in muscle growth are described below.

Fibroblast Growth Factor (FGF)

Both the acidic (FGF1) and basic (FGF2) forms of FGF are capable of stimulating proliferation and inhibiting differentiation in a variety of cells of mesodermal and neuroectodermal origin. FGF2 has been localized in the ECM of skeletal muscle fibers [25, 93] as well as in the myofiber periphery [8]. Furthermore, increased binding of FGF2 antibodies has been documented in damaged and regenerating muscle of human, dog and mouse specimens [7]. Anderson et al. [9] also reported increased FGF staining in regenerating mouse muscles of the SJL/J strain, which possess a superior capacity for new muscle formation compared to other strains.

In examining the effect of the FGFs on the rate of proliferation and fusion of bovine myoblasts, Gospodarowicz et al. [36] found that FGFs purified from bovine pituitary or brain and added to culture media stimulated proliferation and delayed fusion, an activity dependent upon terminal myogenic differentiation. In similar studies, it was shown that FGF2 [3] or bovine pituitary FGF [4] acted as potent mitogens for satellite cells as well. Additionally, Olwin and Hauschka [67] reported that both FGF1 and FGF2 stimulate proliferation and repress terminal differentiation of MM14 myoblasts. These studies strongly suggest that FGF plays a role in regulating skeletal muscle development via stimulation of myoblast proliferation.

Utilizing heparin affinity chromatography to separate the components of CME, Kardami et al. [54] reported the presence of FGF1 in chicken extracts, while Chen et al. [20] reported that mouse CME contained FGF2. Chen et al. [20] reported mouse CME yielded three peaks of mitogenic activity eluting from heparin-agarose columns at NaCl concentrations of 0.4 M, 0.9 M and 2.0 M. The 2.0 M fraction, which eluted similarly to purified FGF2, did not act additively to saturating amounts of FGF2 and was neutralized by anti-FGF2 antibodies, indicating that this mitogenic activity is due to FGF2 or a similar molecule.

Kardami et al. [54] detected activity in chicken muscle extract which eluted between 1.4 and 1.5 M NaCl (characteristic of FGF1) which was capable of stimulating chick myoblast division while inhibiting the onset of fusion and terminal differentiation. The activity was subject to heat, trypsin and acid inactivation, thus sharing many of the attributes of FGF1.

Chen and Quinn [21] and Haugk et al. [44] showed that mouse and bovine CME can act in an additive fashion to saturating concentrations of FGF2 to stimulate myoblast proliferation. Therefore, while CME contains FGF2, within these experiments the mitogenic activity in CME cannot be due solely to FGF2.

Bischoff [15] found that a fraction of rat CME eluting from heparin at 1-2 M NaCl stimulated proliferation of endothelial cells and fibroblasts, as did FGF2, but had no effect on proliferation of satellite cells cultured on single myofibers, confirming other observations that FGF does not activate quiescent satellite cells [13, 51, 52]. However, a low molecular weight component in the rat extract which eluted from heparin at 0-1 M NaCl stimulated the proliferation of rat satellite cells cultivated on single myofibers [15]. Similarly, the 0.9 M NaCl fraction eluted from mouse CME by Chen et al. [20] contained an uncharacterized mitogen for myoblasts and may be identical to the mitogen contained in the 0-1 M NaCl fraction eluted by Bischoff [15]. Hepatocyte growth factor, recently identified as a likely satellite cell activation factor [6], elutes from heparin affinity columns in the 1 M NaCl range [35]. Therefore, the uncharacterized mitogen of Chen et al. [20] and Bischoff [15] eluting from heparin in this range may be HGF.

Platelet-Derived Growth Factor (PDGF)

Platelet-derived growth factor is a potent mitogen for connective tissue cells, including dermal and tendon fibroblasts [74], as well as L6 rat myoblasts [50], cultured chicken myoblasts [92], and cultured porcine satellite cells [23]. Yablonka-Reuveni et al. [90] demonstrated that PDGF significantly promoted C2 muscle cell proliferation and depressed differentiation. Thus, PDGF may play a role in muscle regeneration, increasing the population of myoblasts available for repair following injury. Platelet-derived growth factor can exist as two kinds of homodimers (PDGF-AA and PDGF-BB) or a heterodimer (PDGF-AB). Western blots performed with antibodies against the various forms of PDGF demonstrated the presence of a PDGF-BB-like molecule in a 0.4 M NaCl fraction of mouse CME eluted by heparin affinity chromatography [20].

Transferrin (Tf)

Another possible growth factor component of CME is transferrin (Tf), an iron-binding glycoprotein also found in serum. Tf-like molecules have been isolated from skeletal muscle and whole embryo extracts [45, 54, 59]. Tf has been shown to be important in stimulating muscle cell proliferation and differentiation [79]. Since Tf is detected abundantly in muscle tissue, it is likely to be one constituent of CME. Western blots have shown that Tf is present in chicken muscle extracts [54] and more recently mouse [21] CME. Chen et al. [20] found that anti-Tf antibodies neutralized almost half the mitogenic activity in mouse CME.

Some, but not all, of the mitogenic activity found in stretched chicken muscle extract is also due to a Tf-like factor [84]. Extracts from stretched muscles stimulated chick embryo myoblasts and fibroblasts to a greater extent than the optimum concentration of chick embryo extract, the mitogenic activity of which is due mostly to Tf [45]. Stretched chicken muscle extract also supported the growth of rat myoblasts, suggesting the activity of factors other than Tf, which exhibits specificity for taxonomic class (e.g. aves vs. mammalia) [79]. Kardami et al. [54] also established that chicken muscle extracts are active for chick myoblasts as well as rat myoblasts. The findings of Bischoff [14] and Kardami et al. [54] suggest that factors other than Tf are present in the extracts and that these factors are not species or class-specific.

Bischoff [14] eliminated Tf as the source of activation activity in rat CME. The use of dialysis to remove iron, and presumably Tf activity, from CME did not diminish the mitogenic activity of the CME for rat satellite cells cultivated on single myofibers.

Transforming Growth Factor-β (TGF-β)

TGF-β is a multifunctional polypeptide growth factor consisting of five isoforms. TGF-β3 has been shown to be highly expressed in developing and mature mouse muscle [55], while TGF-β1 shows immunoreactivity in dystrophic muscle specimens [94]. The effects of TGF-β on proliferation may be stimulatory or inhibitory, depending on cell type, growth conditions and the presence or absence of other growth factors [82]. Reports regarding the effect of TGF-β on muscle cell proliferation have been contradictory. Hathaway et al. [43] reported that TGF-β did not inhibit proliferation of cultured ovine satellite cells in serum-containing medium or in serum-free defined medium. However, they also noted that TGF-β significantly suppressed serum-stimulated proliferation of either porcine or bovine satellite cells. Similarly, Allen and Boxhorn [2] demonstrated that TGF-β suppressed proliferation of rat satellite cells. Greene and Allen [37] reported that proliferation of bovine satellite cells was stimulated in a dose-dependent manner by TGF-β in the presence of FGF2. Therefore, TGF-β drastically changes its effects on myoblast growth depending on the presence or absence of a second growth factor. Since it is clear that several growth factors are present in CME, the potential function(s) of TGF-β in this context is unclear.

Haugk et al. [44] found that high concentrations of mouse or bovine CME were required to act additively to a saturating concentration of TGF-β to increase C2 myoblast proliferation, whereas lower concentrations of mouse or bovine CME were unable to produce an additive effect with TGF-β to increase proliferation. Thus higher

concentrations of CME may contain high enough levels of FGF2 or some other growth factor to overcome an inhibitory effect of TGF-β on myoblast proliferation.

Combinations of growth factors

Since the actions of many of the above growth factors change in the presence of a second or perhaps third factor [3, 37], the additivity of CME with saturating doses of several growth factors was examined. Chen and Quinn [21] showed that the addition of CME plus saturating amounts of FGF2, insulin-like growth factor-I (IGF-I), PDGF-BB, epidermal growth factor (EGF), Tf, adrenocorticotrophin (ACTH) and macrophage colony-stimulating growth factor (M-CSF) caused more stimulation of C2 myoblast proliferation than the combination of the seven mitogens without the CME. Similarly, Haugk et al. [44] showed that bovine or mouse CME acted in an additive fashion with a saturating combination of FGF2, IGF-I, IGF-II and TGF-β to increase C2 myoblast proliferation.

The conclusion to be drawn from these studies is that the characterized mitogens FGF1, FGF2, IGF-I, IGF-II, EGF, PDGF, Tf, M-CSF and TGF-β share many properties with CME (Table 1). However, further investigations are needed to fully characterize CME. The additivity experiments presented here do not provide sufficient evidence to include or exclude these growth factors as components of CME.

Differentiation

The current model of myogenic differentiation, derived largely from studies of myogenic cell lines, is essentially a negative regulation model in which mitogenic growth factors (e.g. FGF, PDGF and TGF-β) inhibit the differentiation process [56]. However, in contrast to this interpretation, the insulin-like growth factors (IGF-I and IGF-II) and thyroid hormone (T_3) have been shown to stimulate differentiation, fusion and muscle hypertrophy in a variety of cell types [31, 41, 46, 85, 86]. Thus appropriate stimulation or inhibition of positive external regulators of muscle differentiation could be a strategy to modify skeletal muscle growth.

The CME experiments provide evidence that regenerating muscles not only provide agents which induce satellite cell proliferation, but may also produce agents which induce muscle differentiation. Rat CME effectively stimulated differentiation of myogenic cells from rat embryos [14]. Further evidence for a differentiation-inducing component was noted in an experiment conducted by Summers et al. [84] in which an avian stretched-muscle extract produced a dose-dependent stimulation of creatine kinase (CK) activity in embryonic chick myoblasts. In addition, chicken muscle extracts have been shown to induce substantial myosin heavy chain accumulation in chick myoblasts and rat L6 myoblasts [54]. Recently, Bischoff and Heintz [16] demonstrated effects of crushed muscle extract on regenerating muscle *in vivo*. They reported increased CK activity (an index of skeletal muscle formation) in minced rat muscle after addition of rat muscle extract, suggesting rat CME contains differentiation-inducing activity. In this experiment, it is likely that activation and proliferation factors were introduced to the damaged muscle as well; the use of whole CME and an *in vivo* model makes it difficult to identify which growth factors are involved and what processes they are regulating. The potential components of CME which may be involved in inducing or inhibiting differentiation are explored below.

Insulin-like Growth Factors (IGF-I and IGF-II)

The IGFs are a family of growth-promoting, single-chain polypeptide hormones that are structurally related to insulin [24]. They are often referred to as mitogenic progression factors in that they regulate the progression of fibroblasts through G_1 to the S phase [38]. The IGFs are involved in regeneration of mesodermally-derived tissues and in wound healing [33]. Jennische et al. [49] demonstrated IGF-I immunoreactivity in regenerating rat muscle within 24 h of injury. Additionally, Jennische and Hansson [48] concluded that IGF-I is synthesized in muscle during regeneration due to the high IGF-I immunoreactivity found in myoblasts and myotubes after injection of snake venom. Actions of the IGFs on myogenic cells include inhibition of intracellular muscle proteolysis, stimulation of amino acid uptake, stimulation of myoblast proliferation and promotion of myoblast terminal differentiation [32]. At low concentrations of IGF there is a progressive stimulation of myoblast differentiation, while higher concentrations lead to a decrease in differentiation and a stimulation of proliferation [31].

Chen and Quinn [21] were first to examine whether IGF-I is present in mouse CME and whether it accounts for the full activity of CME. Results showed that a combination of mouse CME with saturating amounts of IGF-I produced a higher level of C2 myoblast proliferation than CME or the saturating concentration of IGF-I alone. Haugk et al. [44] combined mouse or bovine CME with IGF-I and demonstrated that increasing concentrations of either extract acted additively with a saturating concentration of IGF-I to increase C2 myoblast proliferation. Therefore, while IGF-I may be a component of CME, in these experiments it is certainly not responsible for all the mitogenic activity observed.

Most studies have indicated IGF-I and IGF-II exert mitogenic actions via the same receptor, the type-1 IGF receptor [26, 27, 30]. Thus, if IGF-II is a component of CME, its role may be impossible to separate from that of IGF-I. Since CME acts additively with saturating concentrations of both IGFs [21, 44], other factors still may account for the mitogenic activities demonstrated by CME. However, preliminary studies using radioimmunoassays indicate that mouse [21] and bovine (Haugk et al., 1995, unpublished observation) CME contain physiologically significant amounts of IGF-I. It is still unclear whether the IGFs are involved in proliferation, differentiation or both processes within this model system. Furthermore, the presence or absence of another mitogen such as FGF may influence the effects of IGF.

Table 1. Comparison of properties common to crushed muscle extract and known growth factors

	Activates Satellite Cells	Stimulates Proliferation	Stimulates Differentiation
Crushed Muscle Extract	yes	yes	yes
Fibroblast Growth Factor-1	-*	yes	no
Fibroblast Growth Factor-2	yes/no**	yes	no
Hepatocyte Growth Factor	yes	-*	-*
Insulin-like Growth Factor-I	no	yes	yes
Insulin-like Growth Factor-II	no	yes	yes
Platelet-Derived Growth Factor	no	yes	no
Transferrin	no	yes	no
Transforming Growth Factor-β	no	yes/no**	yes/no**

*data unavailable at this time
**results vary with experimental conditions

Transforming Growth Factor-Beta (TGF-β)

TGF-β has been shown to be both a positive and negative regulator of myoblast differentiation. Florini et al. [31] and Olson et al. [66] demonstrated that TGF-β is a potent and persistent inhibitor of L6 and C2 myoblast differentiation. Massagué et al. [58] showed that TGF-β inhibits fusion of established cell lines (L6 and L8 rat skeletal muscle myoblasts) and primary cultures of rat and chick embryo myoblasts. Allen and Boxhorn [2] reported a dose-dependent inhibition of differentiation in response to TGF-β for primary rat satellite cells. However, another study [96] has demonstrated that TGF-β stimulates rather than inhibits differentiation under certain conditions such as mitogen-rich environments.

Presently, little is known about the physiological role of TGF-β during muscle growth *in vivo*. High levels of TGF-β released from platelets at the site of muscle injury may inhibit differentiation in proliferating satellite cells until a sufficient population has been generated. Later in the regeneration process, a decrease in TGF-β and an increase in IGF-I could allow for differentiation and fusion into myotubes [3]. Recently, the expression of TGF-β has been demonstrated in dystrophic muscle specimens [94]. Thus, TGF-β may play a role in the regenerative process. Therefore, TGF-β seems to be a likely component of CME, but the presence of TGF-β in CME has not been confirmed.

Other possible growth factors

A myriad of growth factors and cytokines have been shown to affect proliferation and differentiation of myogenic cells in culture [40]. Of these, leukemia inhibitory factor (LIF), interleukin-6 (IL-6), and other macrophage-released factors are good candidates for components of CME. Macrophages accumulate at the site of muscle injuries, remove necrotic tissue, and attract leukocytes and satellite cells [70]. In addition, macrophage-derived factors are strongly mitogenic for myoblasts [18]. LIF mRNA expression in muscle tissue is induced as early as 12 h after injury, but it is unclear which cells express this message [12]. LIF stimulates the proliferation of primary mouse [11] and human [12] myoblasts in culture, but does not inhibit fusion of these cells into myotubes. LIF also has no effect on the proliferation or differentiation of fibroblasts [11]. When LIF is administered to the site of an *in vivo* muscle injury, it causes an increase in the size of the muscle fibers rather than an increase in fiber number [12]. Additionally, IL-6 has been shown to stimulate cultured mouse myoblast proliferation but to a lesser degree than LIF [11].

The muscle ECM is composed of glycoproteins and proteoglycans, including laminin, fibronectin, hyaluronic acid, heparin, heparin sulfate, collagen and N-acetylglycosamine. Changes in the composition of these components have been documented in muscle regeneration [38]. These molecules have varied effects on muscle cell proliferation and differentiation [38, 40]. Following muscle injury, both bioactive ECM fragments and ECM-bound growth factors (e.g. FGF2, IGFs and TGF-β) could be released via proteases to modify satellite cell activities. Muscle crush injuries cause an increase in certain muscle protease activators [29]. The balance of such proteases, activators and their inhibitors is thought to play an important role in tissue remodelling which occurs following muscle injury [29]. Recently, it was shown that aprotinin, an inhibitor of serine proteases, stimulates differentiation of mouse myoblasts possibly by regulating the activity of TGF-β-like molecules, which are generated from inactive precursors by proteolysis [87]. The presence of bioactive ECM fragments and/or proteases, their activators, or their inhibitors, in CME has not been previously explored.

Generation of reserve myoblasts

Repeatedly injured skeletal muscle still retains its ability to regenerate [61, 78], indicating that a reserve population of satellite cells is retained upon repeated regenerative stress. The mechanism for creating such a population is

unknown at this point. However, evidence for two different models exists. It may be that the two models combine to function cooperatively during muscle growth *in vivo.*

One possible mechanism is that some satellite cells are refractory to activation. Schultz and Heckman-Jones [77] continuously labeled proliferating satellite cells with bromodeoxyuridine for up to 14 days during postnatal muscle growth. A small number of satellite cells remained unlabelled for the entire infusion period. These observations suggest that the satellite cell population can be divided into those that are rapidly dividing and those acting as reserve or "stem" cells. However, it is not known how many mitotic divisions separate a stem cell from a cell available for fusion or how these transitions are regulated. If this mechanism truly occurs *in vivo,* then the use of growth factors, such as those in CME, which clearly contains an "activation" factor, may be utilized to help increase muscle mass in postnatal production animals.

The second possible mechanism is that some activated satellite cells might not undergo differentiation and thus remain as stem cells. Evidence for this model is derived from myoblast transplantation studies in which some implanted myoblasts fused with existing myofibers, but others survived as undifferentiated muscle precursor cells which could be recovered from the implanted muscle and whose progeny could fuse with muscle fibers of a second host [95]. Furthermore, cell cloning and subcloning experiments suggested that chick myogenic "stem" cells exist, which can undergo self-renewing divisions as well as divisions leading to myogenic differentiation [68, 69]. Using mouse myoblast transplantation experiments, Morgan et al. [60] also found evidence for a stem-cell like sub-population, which could give rise to differentiation-competent progeny, and to differentiation-resistant progeny with high self-renewal capacity. If this is the case, then growth factors which could drive more myoblasts to differentiate, analogous to factors such as erythropoietin, granulocyte-macrophage colony-stimulating factor and stem cell factor in hematopoiesis [75, 96], would be useful agents to increase muscle mass via an increase in myoblast contribution to muscle hypertrophy.

Clearly the elucidation of these processes and identification of potential growth factors involved represents an untapped area to be exploited in growth production. Perhaps as with the other processes undergone by satellite cells, local growth factor release from skeletal muscle may regulate the maintenance of this stem cell population.

Conclusions

Recently, cell culture and *in vitro* studies have demonstrated that extracts from injured rat, mouse, chicken or bovine muscle tissue contain potent mitogens for muscle satellite cells and various other established muscle cell lines. The extracts do not appear to act in a species-specific fashion. The precise components of the extracts are currently unknown; however, results from these studies suggest that the extracts contain a combination of known (FGF-like molecule, PDGF-like molecules, IGF-I and Tf) and one or more unknown myoblast mitogens.

In addition to stimulating proliferation of myoblasts which are already in the cell cycle, rat CME, and presumably extracts from other species, is able to activate quiescent satellite cells to enter the cell cycle. This activation is a critical and distinct step in muscle hypertrophy and regeneration. Also, stimulation of differentiation and control of the percentage of reserve myoblasts are important processes in muscle growth and could be potentially influenced by growth factors. However, these areas have received little attention, and need to be investigated further to determine if manipulation of these processes can be exploited to increase meat production.

Each of the processes undergone by satellite cells is distinctly regulated by different growth factor combinations. Crushed muscle extract studies provide a model system to analyze the factors involved in satellite cell activation and postnatal muscle growth. Because the muscle regeneration process is comparable to embryonic muscle development, it is probable that some of the same factors present in CME are also present in varying concentrations in maturing muscles of young animals. The identification, isolation and large-scale production of the naturally-occurring myoblast activation factor which is clearly present in CME could greatly benefit research and perhaps clinical and commercial applications. It is evident that continued research is needed to investigate the role of growth factors in muscle growth and regeneration.

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