An Angiogenic Extract from Skeletal Muscle Stimulates Monocyte and Endothelial Cell Chemotaxis *In Vitro* (43282)

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Abstract. **The purpose of this study was to determine whether the extraction of skeletal muscle with a combination of ethanol and hydrochloric acid yields a product capable of stimulating angiogenesis. The resulting extract stimulated inflammation in the rabbit corneal assay, which was followed by capillary formation. In order to determine whether the observed angiogenesis was stimulated by a factor(s) acting directly on the endothelial cells versus a factor(s) recruiting macrophages that in turn release factors acting on endothelial cells, the muscle extract was tested for endothelial cell and monocyte chemotaxis activity *in vitro*. The muscle extract stimulated significant endothelial cell chemotaxis at concentrations between 94 and 750 μg of protein/ml and significant monocyte chemotaxis at concentrations between 8 and 75 μg of protein/ml. Polyacrylamide gel electrophoresis suggests that basic fibroblast growth factor and transforming growth factor-β may be present in this acid/ethanol extract of skeletal muscle.**
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When a muscle is damaged it undergoes a characteristic sequence of changes as it degenerates and subsequently regenerates (1, 2). Forty-eight to 72 hr after the insult, a large number of macrophages appear on the surface of the muscle. Concomitant with this cellular influx is the appearance of a capillary network. With time, the inflammatory cells and the blood vessels penetrate the periphery of the muscle and move centripetally. Subsequently, the macrophages devour the necrotic muscle fibers, and the satellite cells, the precursors for regenerating muscle, begin to proliferate. Satellite cells fuse to form myotubes, which mature into muscle fibers when the muscle becomes reinnervated. The regeneration and revascularization processes reach the center of muscles with small diameters, such as the extensor digitorum longus muscle of the rat. In muscles with larger diameters, such as the palmaris longus muscle of the monkey, only the periphery regenerates and the center becomes filled with a dense connective tissue scar (3). A viable satellite cell population, reinnervation, and revascularization are the critical requirements for muscle regeneration. The speed and degree of revascularization may be responsible for the failed regeneration of larger muscles (4). In order to augment the revascularization response, the stimulus for the revascularization must be determined. In a recent report, we (5) showed that an extract derived from damaged skeletal muscle contains a soluble factor(s) that stimulates dose-dependent, inflammation-associated angiogenesis in the rabbit cornea. At the time, we hypothesized that the angiogenesis was indirect. That is, the damaged muscle recruits macrophages which are capable of releasing factors that elicit the formation of capillaries from endothelial cells (6, 7). To test this theory further, we developed a new procedure for extracting skeletal muscle and tested the extract for angiogenic activity in the rabbit cornea and for monocyte and endothelial cell chemotaxis *in vitro*. The migration of endothelial cells toward a chemical gradient is one of the components of angiogenesis. If a compound is capable of stimulating direct angiogenesis, it should attract endothelial cells *in vitro*. If a compound is an indirect angiogenesis factor, it should attract monocytes *in vitro*.

Materials and Methods

Donor Muscle. The rabbits used in this study were New Zealand White females (1.8–2.7 kg) maintained

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at the University of Minnesota. The animals were sacrificed by intracardiac injection of sodium pentobarbital (23 mg/kg). Muscles were harvested from the hind limbs by severing them from their tendons of origin and insertion. No attempt was made to discriminate between fast and slow muscles.

Extract Preparation. Extract was prepared according to Roberts *et al.* (8). Ten grams of donor muscle were minced into 1–2-mm^2 pieces in a solution of 39.2 ml of absolute ethanol, 0.78 ml of concentrated hydrochloric acid, 3.4 mg of phenylmethylsulfonyl fluoride, and 0.2 mg of pepstatin. The volume was adjusted to 60 ml with distilled water and the solution was stirred overnight at 4°C. The extract was centrifuged at 2000 rpm for 15 min and the residue was re-extracted for 2 hr at 4°C with 31 ml of 95% ethanol, 8.6 ml of distilled water, and 0.6 ml of concentrated HCl. Sodium acetate buffer (0.8 ml, pH 5.3) was added to the combined supernatants and the pH was adjusted to 5.3 with concentrated ammonium hydroxide. Two volumes of anhydrous ethanol and 4 vol of anhydrous ether were added, and the resulting mixture was allowed to sit at −20°C for 48 hr. The extract was centrifuged at 5000 rpm for 30 min and the supernatant was discarded. The pelleted precipitate was redissolved in 1 *M* acetic acid and centrifuged again (3000 rpm, 15 min) to remove any insoluble material. The supernatant was dialyzed against 0.17 *M* acetic acid at 4°C in 2000 mol wt cutoff tubing for 72 hr with three changes of acetic acid. The extract was then lyophilized and stored at −20°C.

Incorporation of Extract into Hydron. Ten percent Hydron (Hydromed Sciences, NJ) was prepared in 70% ethanol with the addition of 1% polyethylene glycol. Hydron was mixed 1:1 with the test sample and 20-μl aliquots were pipetted onto a sterile plastic-coated surface. The Hydron/extract was dried under vacuum for at least 2 hr prior to implantation in the rabbit cornea. Doses of 160 and 360 μg of protein/implant were tested (5).

Corneal Assay for Angiogenesis. After the Hydron/extract was dried, it was assayed for angiogenic activity in the rabbit cornea (9) ($n = 6$ corneas for the controls and each concentration tested). Rabbits were anesthetized with a ketamine (44 mg/kg)-acepromazine (1:1) solution. In addition, the topical anesthetic proparacaine hydrochloride was administered to the cornea. The eye of the rabbit was displaced from the orbit with a petite-point Allis forceps and an incision, approximately half the thickness of the cornea, was made in the center of the cornea with a microsurgery scalpel blade. A pocket was created between the layers of the corneal stroma with a Castroviego cyclodialysis spatula, starting at the incision and terminating 1–2 mm from the capillary bed at the corneal-scleral limbus. The test pellet was inserted into the mouth of the micropocket and pushed to the end. Both eyes received the same test material. After surgery, each eye was given several drops of Neosporin ophthalmic antibiotic. The corneas were examined on a daily basis for 1 week for the growth of new blood vessels from the corneal-scleral limbus directed at the implant. Vascular responses were graded as +1 for vascular sprouts directed toward the implant, +2 for new capillaries extending to half the distance between the limbus and the implant, +3 for capillaries reaching the base of the implant, and +4 for the vessel growth into and around the test pellet. Controls consisted of 0.1 *M* acetic acid in Hydron.

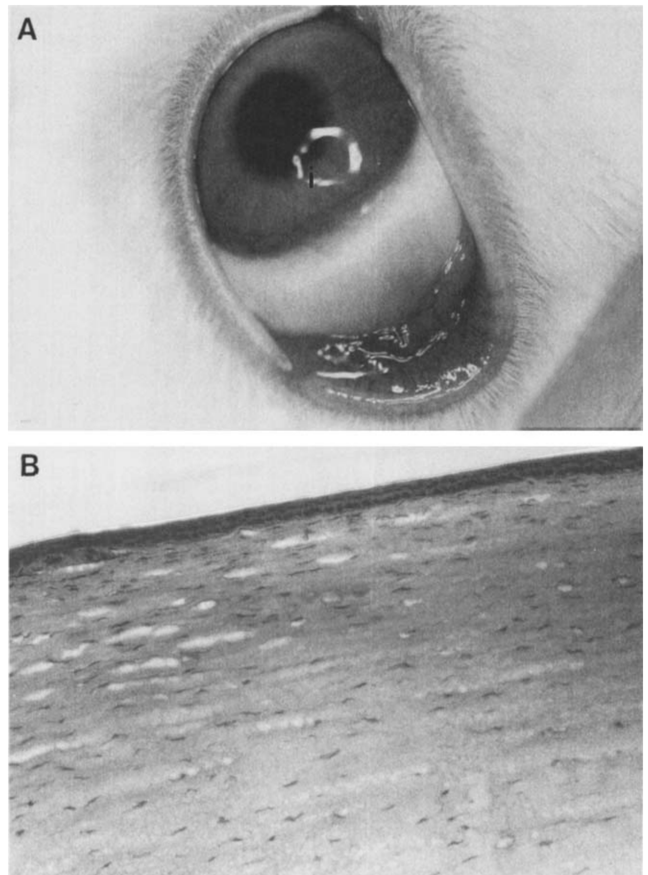

Figure 1. Control corneal implant 7 days after implantation (i) (0.1 *M* acetic acid in Hydron). (A) Gross photograph showing no vascular response to the implant (original magnification ×4). (B) Cross-section of the cornea in (a), confirming the absence of blood vessels in the corneal stroma (hematoxylin and eosin stain; original magnification ×100).

The Culture of Rabbit Wound Capillary Endothelial Cells. Rabbit wound capillary endothelial cells were isolated and cultured according to a modification of a previously published method (10). Small (2 in × 2 in) polyvinyl alcohol sponges soaked in sterile 0.9% saline were implanted subcutaneously in the backs of rabbits. After 14 days the sponges were removed, minced, and digested with an enzyme cocktail of collagenase-DNase-protease. The cell suspension was then layered on a discontinuous Percoll gradient and endothelial cells were collected from the 30 to 50% interface. These cells

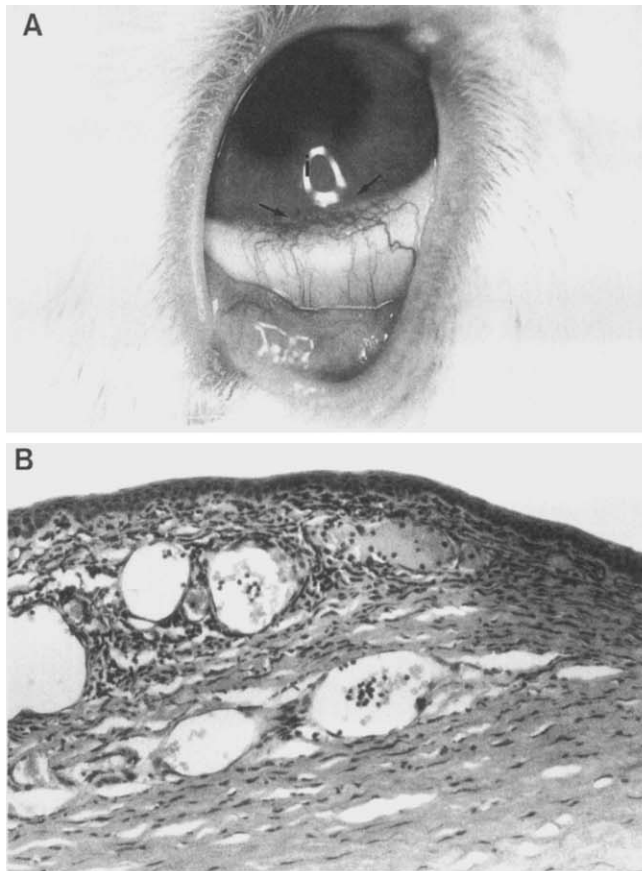

Figure 2. Rabbit cornea with an implant (i) of 200 μg of muscle extract in Hydron, 2 days after surgery. (A) Gross photograph showing corneal opacification (arrows) between the limbus and the implant (original magnification ×4). (B) Cross-section of the cornea in (A), showing an influx of inflammatory cells (hematoxylin and eosin; original magnification ×100).

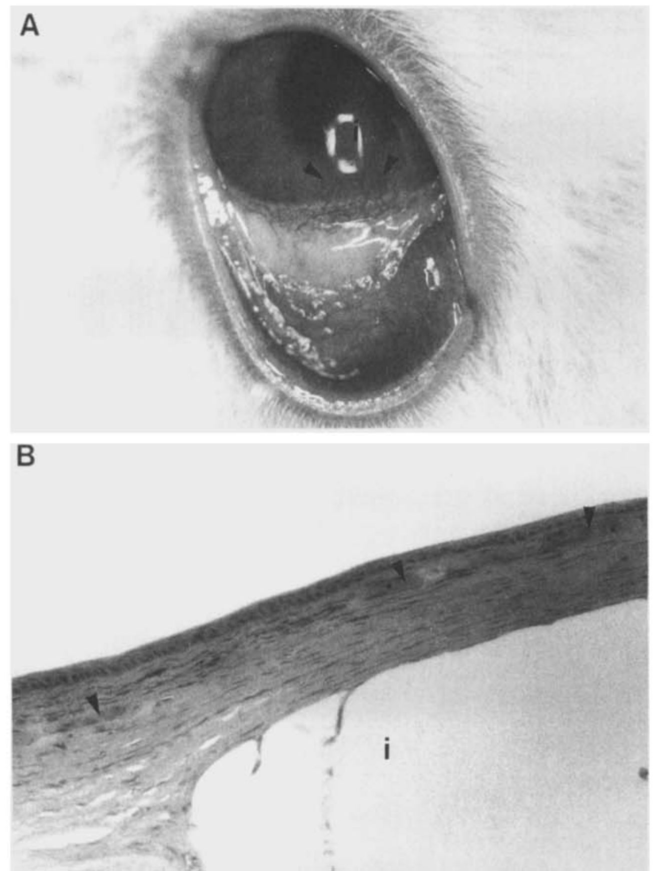

Figure 3. Rabbit cornea with an implant (i) of 200 μg of muscle extract in Hydron, 7 days after implantation. (A) Gross photograph showing the new capillaries (arrowheads) formed between the limbal blood vessels and the implant (original magnification ×4). (B) Cross-section of the cornea in (A) showing capillaries (arrowheads) in the corneal stroma (hematoxylin and eosin; original magnification ×100).

were cultured on Matrigel coated tissue culture flasks in Media 199 (M199) supplemented with 10% fresh rabbit serum and 5% fetal calf serum. These cells stained positive with fluorescent, acetylated, low-density lipoprotein, and the cultures contained 95% endothelial cells. Cultures with a significant amount of contamination by other cell types (<85% positive low-density lipoprotein staining) were discarded. These cells were used until their third passage.

Endothelial Cell Chemotaxis Assay. The acid/ethanol muscle extract was tested for endothelial cell chemotaxis in a modified Boyden chamber assay (11) ($n = 3$ separate tests, narrowing the range of concentrations with each test). Twenty-four hours prior to use, the endothelial cells were rinsed two times with Hanks' balanced salt solution and the culture medium was replaced by M199 with 0.2% lactalbumin. On the day of the experiment, the cells were detached from the tissue culture flask with an enzyme cocktail (0.2% DNAse and 0.5% collagenase in M199), rinsed with Hanks' balanced salt solution, resuspended in M199, and counted with a hemocytometer. Polycarbonate filters (80 × 25 mm, 8-μm pores; Nuclepore, Pleasanton, CA) were precoated with fibronectin (1 μg/ml). A range of concentrations of muscle extract (11–1500 μg protein/ml) was placed in the bottom wells, in quadruplicate, of a 48-well chemotaxis chamber (Neuroprobe, Cabin John, MD) and the filter was positioned between the bottom and top wells. The endothelial cells were then pipetted into the upper wells (3.5×10^4 cells/well). The chamber was incubated at 37°C in a humidified, 5% CO_2 atmosphere for 4 hr, at which time the nonmigrating cells were wiped off the upper surface of the filter. The filter was stained with Wright's stain and the migrating cells were counted and compared with the controls. M199 with 0.2% lactalbumin served as the negative control and platelet-derived wound-healing formula (12) served as the positive control. The results were analyzed with a two-tailed Student's t test.

Isolation of Peripheral Blood Monocytes. Sixty milliliters of blood were drawn from healthy volunteers and centrifuged at 1200 rpm for 20 min. The buffy coat was removed, pipetted over a Histopaque (Sigma Chemical Co., St. Louis, MO) gradient, and centrifuged

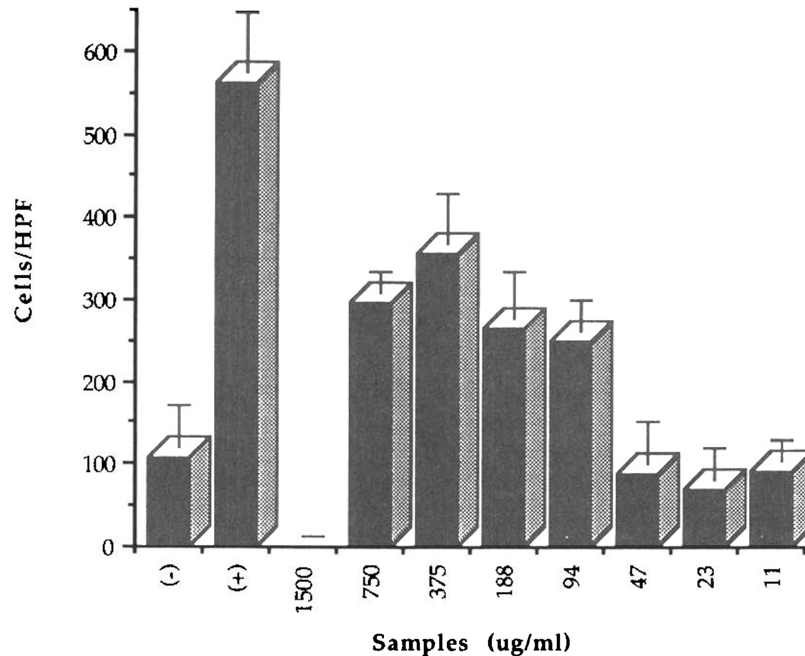

Figure 4. Endothelial cell chemotaxis. At concentrations of 94, 188, 375, and 750 μg of protein/ml, the muscle extract stimulated significantly more chemotaxis than the negative control ($P < 0.005$). There were no significant differences between the number of cells stimulated to move by these individual concentrations. The error bars represent SD.

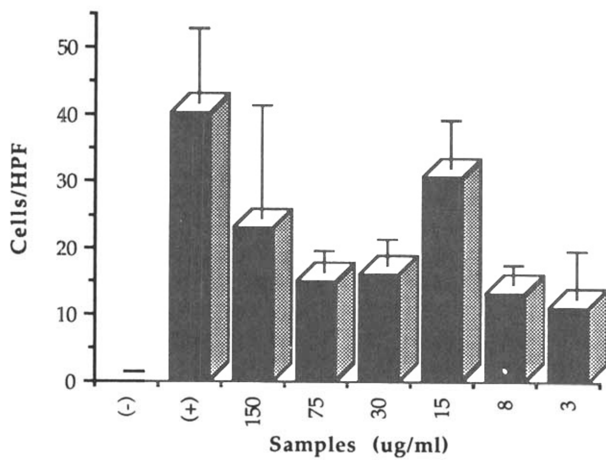

Figure 5. Monocyte chemotaxis. At concentrations of 8, 15, 30, and 75 μg of protein/ml, the muscle extract elicited significantly more chemotaxis of peripheral blood monocytes than the negative control ($P < 0.0005$). The number of cells stimulated to migrate by the 15-μg/ml concentration is significantly higher than the 8 μg/ml ($P = 0.003$) and 30-μg/ml ($P = 0.01$) concentrations. The error bars represent SD.

at 1500 rpm for 30 min. The monocytes were located in the cloudy layer at the interface. The monocytes were removed and rinsed three times in Dulbecco's modified Eagle's media. The final pellet was resuspended in Dulbecco's modified Eagle's media with 1% human serum albumin for the chemotaxis assay. The percentage of monocytes was determined by staining a cytospin preparation with myeloperoxidase.

Peripheral Blood Monocyte Chemotaxis Assay. The assay ($n = 3$ separate tests, narrowing the range of concentrations with each test) was similar to that used for endothelial cells, with the following exceptions. The filters contained 5-μm pores, 5×10^4 monocyte/well were used, and the incubation was in a 10% CO_2, 37°C, humidified atmosphere for 90 min. The migrating cells were stained with myeloperoxidase to distinguish monocytes from neutrophils and lymphocytes. The concentrations tested ranged from 3 to 150 μg protein/ml. The results were analyzed with a two-tailed Student's t test.

Polyacrylamide Gel Electrophoresis. Electrophoresis of 15% polyacrylamide gels was carried out according to the methods of Laemmli (13). Gels were fixed in methanol and acetic acid and silver-stained (Bio-Rad).

Results

Corneal Assay of Angiogenesis. The controls, corneas receiving 0.1 M acetic acid in Hydron, did not exhibit visible corneal opacification 2–3 days after surgery and failed to stimulate any capillary formation by 7 days (Fig. 1). Implants of 200 μg and 400 μg of muscle extract stimulated corneal opacification 2–3 days after implantation, which was followed by +3 and +4 angiogenesis by 7 days (Figs. 2 and 3).

Endothelial Cell Chemotaxis. The negative control, M199 with 0.2% lactalbumin, caused the migration of 108 cells/high-powered field. The positive control, platelet-derived wound-healing formula (12) stimulated an average of 561 ± 74 cells/high-powered field

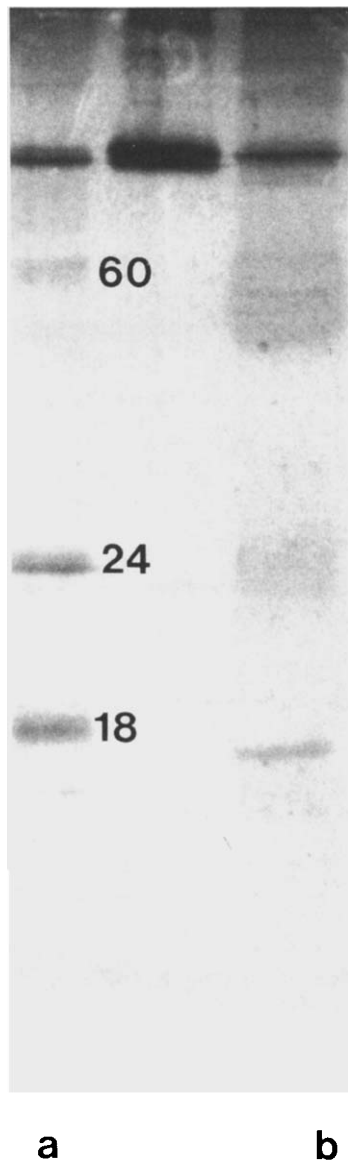

Figure 6. Polyacrylamide gel electrophoresis. Lane a contains the molecular weight standards (60 kDa, 25 kDa, 18 kDa). Lane b contains the muscle extract. The muscle extract exhibits a broad band in at 60,000 mol wt, a broad band around 24,000 mol wt, and a sharper band just below the 18,000 mol wt standard.

to migrate across the filter. Concentrations of 94, 188, 375, and 750 μg of protein/ml were effective in eliciting the chemotaxis of a significantly higher number of cells than the negative control ($P < 0.005$). There was no significant difference in the chemotaxis stimulated by these concentrations. The results are summarized in Figure 4.

Peripheral Blood Monocyte Chemotaxis. The negative control, Dulbecco's modified Eagle's media plus 1% human serum albumin, failed to stimulate the chemotaxis of any monocytes. In response to platelet-derived wound-healing formula, the positive control, 40 ± 11 cells/high-powered field migrated across the filter. Concentrations of 8, 15, 30, and 75 μg of protein/ml elicited the chemotaxis of a significantly higher number of cells than the negative control ($P < 0.0005$). The number of cells responding to the 15-μg of protein/ml concentration was statistically the highest when compared with the 8-μg/ml ($P = 0.003$) and 30-μg/ml ($P = 0.01$) concentrations. The results are summarized in Figure 5.

Polyacrylamide Gel Electrophoresis. This acid/ethanol-extraction procedure yields a solution that exhibits three bands on a polyacrylamide gel. One broad band appears in the 60,000 mol wt region. A second broad band migrated the same distance as the 24,000 mol wt standard, and a sharp band is seen just below the 18,000 mol wt standard, approximately 14,000 mol wt (Fig. 6).

Discussion

The results of this study confirm a prior report from this laboratory that skeletal muscle releases a soluble factor(s) capable of stimulating angiogenesis (5). An acid/ethanol extract from skeletal muscle stimulated angiogenesis in the rabbit cornea. Visual inspection and histological examination revealed that the neovascularization was preceded by an influx of inflammatory cells. This procedure also yielded a partially purified product with the capacity to stimulate the chemotaxis of endothelial cells and monocytes *in vitro*.

In the previous report (5), the muscle was extracted with phosphate buffered saline, followed by filtration with an Amicon filter (PM30). The resulting extract stimulated corneal angiogenesis, but failed to elicit endothelial cell or monocyte chemotaxis *in vitro*. This was not surprising, however, due to the appearance of 30–40 bands when this extract was run on a polyacrylamide gel (unpublished results). The extraction procedure reported in this paper eliminated a large number of proteins that may have been inhibiting the action of the endothelial cell and monocyte chemoattractants.

The invasion of inflammatory cells into the cornea upon implantation of the muscle extract was not unexpected. When a muscle is freely autografted, an early event in its degeneration and subsequent regeneration is an invasion of macrophages and neutrophils (14). As the inflammatory cells move to the center of the graft, the formation of new capillaries appears to follow. The regenerating muscle fibers are closely associated with these new vessels. This pattern suggests an indirect angiogenic mechanism. A soluble factor(s) from damaged skeletal muscle recruits macrophages and neutrophils, which in turn release factors inducing endothelial cell proliferation, migration, and tube formation. The ability of the muscle extract to attract monocytes *in vitro* is consistent with this theory.

The acid/ethanol extract derived from skeletal muscle is also capable of stimulating endothelial cell chemotaxis. This result suggests that a direct angiogenic

mechanism is at work. Polyacrylamide gel electrophoresis of the extract provides a possible explanation. Two of the bands on the gel coincide with the molecular weights of two reported angiogenesis factors. The band at 24,000 mol wt may be transforming growth factor-β (TGF-β). TGF-β induces indirect angiogenesis in the rabbit cornea (15) and when implanted subcutaneously in neonatal mice (16). In addition, Wahl *et al.* (17) have demonstrated TGF-β's ability to stimulate monocyte chemotaxis *in vitro*. The band at 14,000–16,000 mol wt roughly coincides with basic fibroblast growth factor (bFGF). Basic FGF has been reported by a number of investigators to stimulate direct angiogenesis *in vitro* (18–23), and endothelial cell proliferation (20, 22, 24–27), migration (25, 26), and tube formation *in vitro* (28, 29). In our hands, however, bFGF fails to stimulate angiogenesis in the absence of inflammation (30). If TGF-β is present in the muscle extract, it may induce enough inflammation to activate bFGF's angiogenesis activity.

Once the identities of the factors stimulating the revascularization in regenerating muscles are determined, we may be able to increase the concentration of these endogenous factors in large nonregenerating muscles by intramuscular injection or implantation of slow-release polymers and/or osmotic pumps. The proper balance of these compounds or exogenous angiogenic/growth factors administered over the appropriate period of time may improve the speed and degree of the revascularization and improve the accompanying regeneration.

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