Angiogenic Activity in Damaged Skeletal Muscle (43025)

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Abstract. **After a muscle is damaged, blood vessels spontaneously grow into the injured region as the muscle fibers regenerate. The stimulus for this vascular ingrowth is currently unknown. We hypothesized that the damaged muscle releases a factor(s) capable of stimulating this revascularization. To test this theory, extracts were prepared from rabbit hind limb muscles and incorporated into Hydron, a slow-release polymer. Pellets of the extract containing Hydron were implanted between the layers of the rabbit corneal stroma as an assay for angiogenic activity. The normally avascular corneas were examined 7 days after surgery for the presence of new blood vessels. Skeletal muscle-derived extract from rabbits elicited positive angiogenic responses in a dose-dependent manner. Four hundred to 500 μg of the skeletal muscle-derived extract were required to produce maximum vessel ingrowth. The control, Dulbecco's phosphate-buffered saline in Hydron, failed to stimulate neovascularization.**

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Spontaneous revascularization in a muscle damaged by free grafting begins 2 to 3 days postoperatively, regardless of whether the muscle is grafted intact (1, 2) or as minced fragments (3, 4). A network of capillaries originating from surrounding tissues forms on the surface of the graft, penetrates the periphery of the muscle, and proceeds centripetally, creating new vascular pathways as well as utilizing the basal laminae of original vascular channels (2). Concomitant with this vascular ingrowth is a substantial infiltration of macrophages and activation of myogenic cells surviving in the periphery of the graft. Macrophages phagocytize the debris associated with the breakdown of necrotic muscle fibers in the graft, and the myogenic cells serve as the precursors for regenerating muscle fibers. In small muscle grafts, such as the extensor digitorum longus muscle of the rat, new vascular ingrowth and the regenerative process reach the center of graft in a matter of days. In contrast, revascularization of larger muscle grafts (>3 g), such as the palmaris longus muscle of the monkey, is so much slower relative to the size of the graft that the new vessels and regenerating muscle fail to reach the center of the graft before it is replaced with a core of dense connective tissue (5). The spatial and temporal relationships between ingrowing blood vessels and regenerating muscle fibers suggest a crucial role for revascularization in the successful regeneration of free muscle grafts.

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Although the pattern of revascularization has been described in detail, the stimulus for this revascularization has yet to be determined. To investigate this question, Phillips (6), in preliminary experiments, implanted fragments of rat skeletal muscle into the rat cornea to see if they would stimulate the growth of blood vessels into the normally avascular cornea (7). The muscle tissue elicited the initial stages of an angiogenic response, but as the tissue broke down, the new vessels regressed. To provide a more sustained release of the soluble factors within muscle, an extract derived from rat skeletal muscle was incorporated into Elvax, a slow-release polymer, and implanted in the rat cornea. In all cases angiogenesis was stimulated (6). Unfortunately, due to the size of the rat cornea, the amount of extract incorporated into the Elvax was difficult to quantitate and the responses were only recorded as positive or negative.

To test the hypothesis that damaged skeletal muscle releases a factor(s) capable of stimulating angiogenesis, an extract was prepared from injured skeletal muscle of rabbits according to the method of Bischoff (8), incorporated into Hydron, a slow-release polymer (9), and implanted in the corneal stroma of the rabbit as an assay for angiogenic activity (10).

Materials and Methods

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

tained at the University of Minnesota. Rabbits were euthanized with intracardiac injection of sodium pentobarbital (23 mg/kg). Hind limb muscles were removed by cutting the muscles from their origins and insertions. Muscles were chosen randomly, with no attempt made to discriminate between fast and slow muscles.

Extract Preparation. Extract from the source of muscle described above was prepared according to the method of Bischoff (8). Ten to 12 g of donor tissue were crushed in 50 ml of Dulbecco's phosphate-buffered saline (DPBS) and stirred for 1.5 hr at 4°C. Tissue fragments were removed with forceps and the extract was cleared of debris and red blood cells by centrifugation at 20,000 rpm for 60 min. The supernatant was concentrated 6- to 10-fold with an Amicon PM30 ultrafiltration membrane (M_r 30,000 cutoff). The resulting extract was dialyzed for 48 hr against double-distilled H_2O (M_r 12,000–14,000 cutoff dialysis tubing) and lyophilized. Extract was dissolved-suspended in DPBS prior to incorporation into Hydron.

Incorporation of Extracts into Hydron. The Hydron polymer was prepared by dissolving 10% Hydron in 70% ethanol (1 g/10 ml). When the Hydron was in solution, 1% polyethylene glycol was added. Hydron was then mixed 1:1 with the test sample. Twenty-microliter aliquots were pipetted onto a plastic-coated surface and dried under slight vacuum for at least 2 hr before implantation. The doses of extract tested ranged from 10 to 500 μg.

Hydron was used to assure a sustained release of the muscle extracts. In earlier experiments, a small piece of skeletal muscle was implanted into the cornea. The muscle did stimulate the initial stages of an angiogenic response, but with time the muscle tissue broke down and the newly formed vessels regressed (unpublished observations). Other work has shown that angiogenic stimuli must persist in order to maintain capillary ingrowth (11). An extract-laden Hydron pellet implanted in the rabbit cornea is similar to a damaged muscle. In both cases, a source is releasing factors which may or may not stimulate the directed growth of blood vessels.

Corneal Assay for Angiogenesis. Once incorporated into Hydron, each extract was tested for angiogenic activity in the corneal micropocket assay (10). Rabbits were anesthetized with a 1:1 mixture of ketamine:acepromazine at a concentration of 44 mg/kg. Plus, a few drops of the topical anesthetic proparacaine hydrochloride were administered to the cornea. Next, the rabbit's eye was brought out of the socket with a petite point Allis forceps, and an incision was made in the center of the cornea with a microsurgery scalpel blade. The incision was approximately one half of the corneal thickness in depth. Starting at the incision, a micropocket was created between the collagenous layers of the corneal stroma with a Castroviego cyclodialysis spatula. This pocket extended to a point 1–2 mm from the capillary bed at the corneal-scleral limbus. The Hydron pellet containing the extract to be tested was inserted into the mouth of the micropocket and pushed to the end. Both eyes of the rabbit received implants containing the same extract. After surgery, the eyes received several drops of Neosporin ophthalmic antibiotic. The eyes were examined visually for 1 week on a daily basis for any direct growth of capillaries toward the pellet. Blood vessels growing in the cornea were recorded as a positive response on a graded scale; +/− for undirected vascular activity, +1 for new capillaries directed toward the implant, +2 for capillary growth half the distance to the implant, +3 for capillaries reaching the implant, and +4 for capillaries growing into and around the implant. Seven days after surgery, the eyes were photographed and the animals were sacrificed. The eyes were subsequently removed, fixed in 10% formaldehyde in DPBS, sectioned at 7 μm, and stained with hematoxylin and eosin for histologic examination. The areas of neovascular responses to each extract concentration were measured from representative photographs using a digitizing tablet (Zidas; Carl Zeiss, Inc.). Each photograph was projected onto the tablet and the perimeter of the blood vessels within the cornea was traced with the cursor three times. Data from the same concentrations of extract were pooled and the program generated the mean area and standard deviation for each concentration.

After comparing the available assays for angiogenesis, the corneal assay and Hydron combination was chosen for simplicity and consistency in interpreting experimental results. In the corneal assay, the test substance is initially placed in an avascular environment. In contrast, in the chick chorioallantoic membrane assay (12) and the hamster cheek pouch assay (13, 14), the material to be assayed is placed on preexisting vessels. Therefore, formation of capillaries in the avascular cornea represents true neovascularization versus rearrangement of preexisting vessels, making it easier to visualize positive angiogenic responses. Furthermore, Jakob *et al.* (15) and Ryan and Stockley (16) performed extensive experiments in which a variety of release vehicles alone (Millipore filters, glass fibre discs, gelatine and viscose sponges, discs of filter paper, agarose, and polyacrylamide gel) as well as natural egg components (egg shell membrane, coagulated albumin, and coagulated yolk) gave inflammation-associated positive reactions on the chick chorioallantoic membrane.

Results

Visual inspection revealed that the crude extracts from rabbit skeletal muscle stimulated a dose-dependent characteristic angiogenic response in the rabbit cornea. Maximum (+4) reactions were consistently elicited by 400–500 μg of muscle extract (Fig. 1). One to 2 days following implantation in the cornea, the vessels at the corneal-scleral limbus were dilated. Two to 3 days postoperatively, a cloudy white halo surrounded

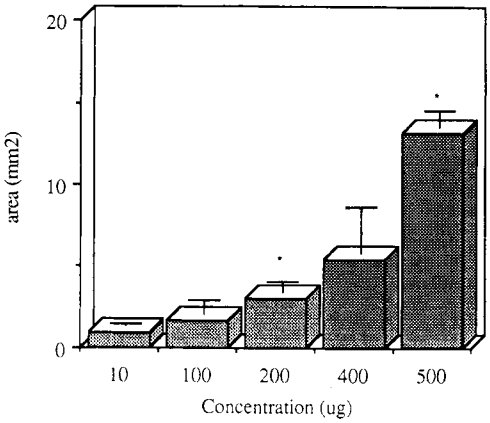

Figure 1. This graph represents the area of the angiogenic responses to various concentrations of rabbit skeletal muscle extract. *The values for 200 and 500 μg of rabbit muscle extract are significantly higher than the baseline value of 10 μg at the 0.90 confidence level using a two-tailed Student's t test. The value for 400 μg of rabbit muscle extract is not significantly higher than the 10 μg value due to the large variation in the sample numbers. Thus, there is a general trend toward dose dependence.

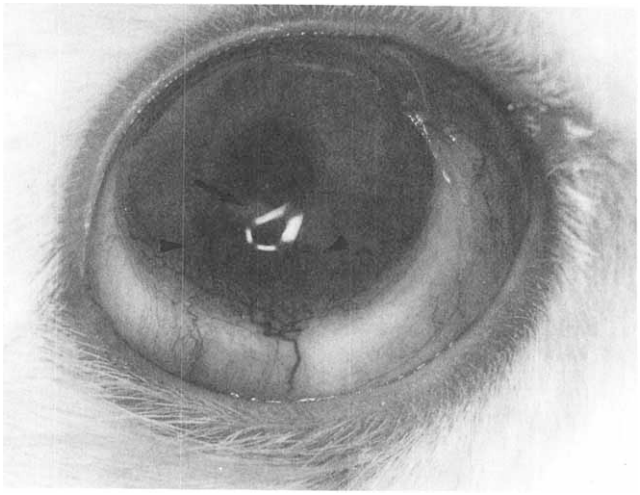

Figure 2. Rabbit eye with a +4 angiogenic response to an implant (arrow) of 500 μg of extract derived from rabbit skeletal muscle. New capillaries (arrowheads) have grown up to and around the implant (original magnification ×4).

the implant and tiny vessel loops oriented toward the implant formed in the cornea. After 4 and 5 days, small capillary sprouts arose from the vessel loops and extended to the base of the implant. By days 6 and 7 new capillaries had progressed centripetally and converged on the implant (Fig. 2). DPBS in Hydron failed to elicit a vascular response (Fig. 3).

Histologically, implants demonstrating positive angiogenic reactions were characterized by a distinct cellular response (Fig. 4). Macrophages and neutrophils were observed in the corneal stroma adjacent to the implant and in the implant itself when it was retained in the section. The infiltration of inflammatory cells reached its peak 2–3 days after implantation (Fig. 5). The control implants attracted only a few macrophages

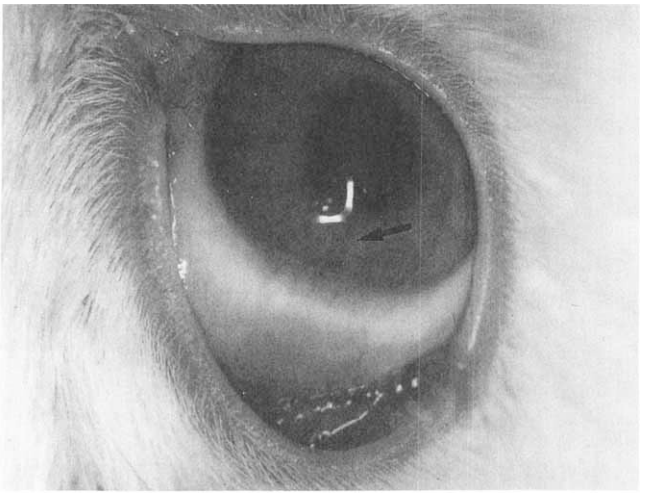

Figure 3. Rabbit cornea with negative angiogenic response to the control, DPBS in Hydron. The implant (arrow) failed to stimulate neovascularization (original magnification ×4).

and neutrophils, and only an occasional capillary loop was observed in the corneal stroma (Fig. 6). The results are summarized in Figure 1.

Discussion

The results of this study clearly demonstrate that an angiogenesis factor(s) is released from skeletal muscle upon injury. This strongly supports the hypothesis that damaged skeletal muscle is capable of stimulating its own revascularization as it regenerates. Furthermore, microscopic evaluation of corneas showing positive and negative angiogenic responses provided insight into the mechanism behind the observed angiogenesis in both the cornea and injured skeletal muscle. Without exception, positive angiogenic responses in the cornea were characterized by an inflammatory response. Macrophages and neutrophils were seen in the corneal stroma around the implant, between the implant and the limbal blood vessels, and within the implant itself. In contrast, these cellular reactions were tiny or nonexistent in those corneas with negative angiogenic responses. Histologic examination of corneal implants over time demonstrated that inflammatory cells invade the corneal stroma prior to vascular ingrowth. These results suggest that extracts derived from injured muscle are chemotactic for macrophages and neutrophils. Once in the cornea, activated macrophages (17–22) and neutrophils (23–27) have the capacity to stimulate endothelial cell proliferation and migration by the release of their own products.

This sequence of events observed in the corneal micropocket assay is similar to the sequence of events observed following the ischemic injury that occurs after free whole muscle transplantation (28). This cellular infiltration is closely associated with the first sign of new capillaries on the surface of the graft. As the muscle regenerates, it seems as though the inflammatory cells lead the new blood vessels and the regenerative process

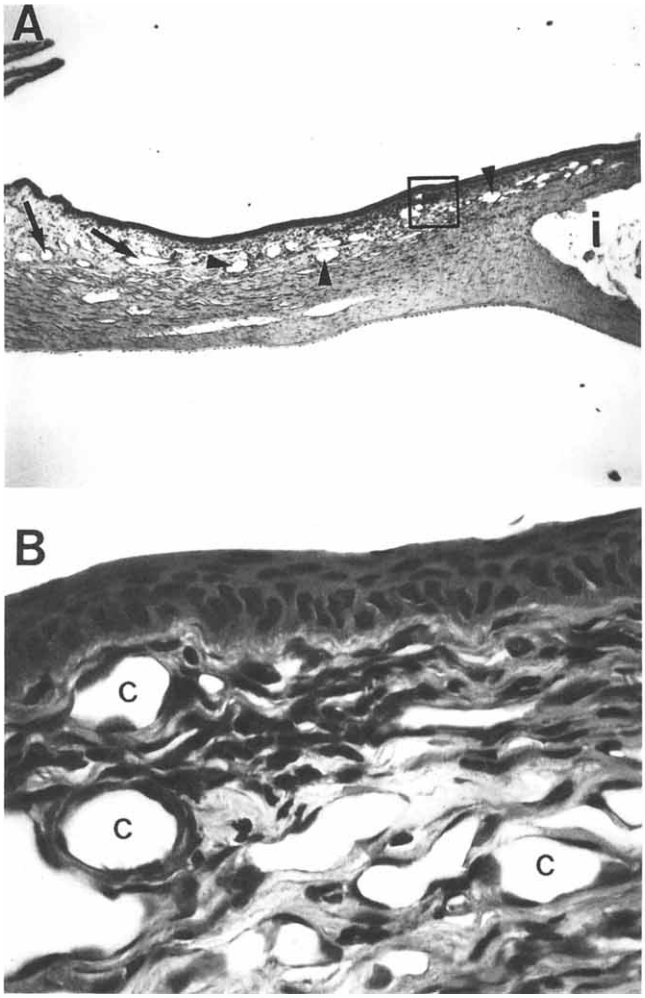

Figure 4. A, Histologic section of a cornea with a +4 angiogenic response to an implant (i) of 500 μg of extract derived from rabbit skeletal muscle. New capillaries originating at the limbal blood vessels (arrows) have grown through the corneal stroma to the implant (original magnification ×50). *B*, High magnification photograph of the boxed region in A demonstrating the presence of vessels (c) lined with endothelium (H & E; original magnification ×600).

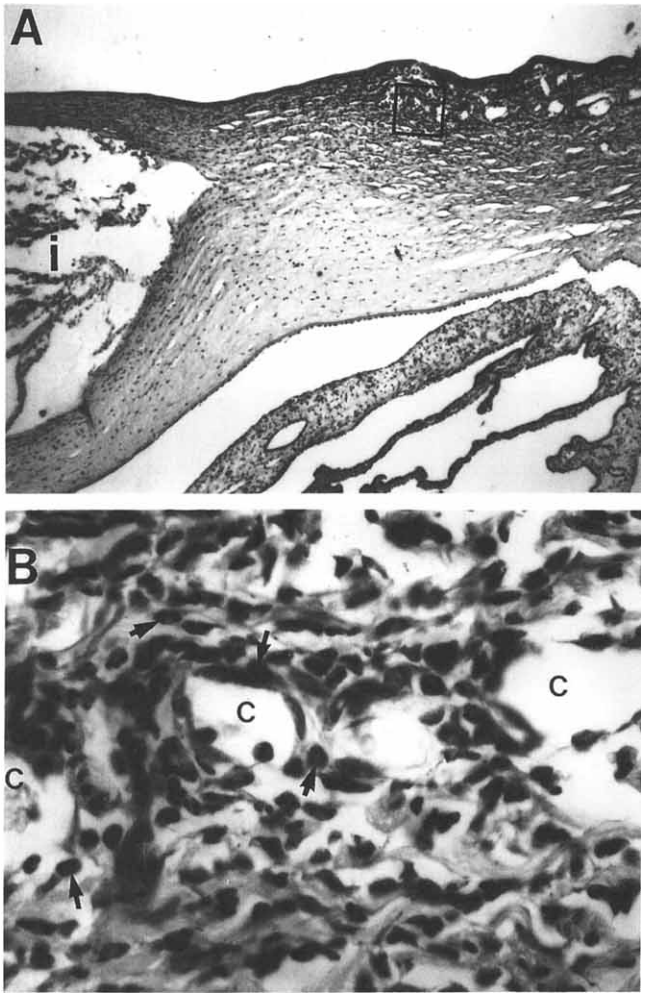

Figure 5. A, Histologic section of a rabbit cornea showing the inflammatory reaction, 2 days after surgery, to an implant (i) of 500 μg of extract derived from rabbit skeletal muscle (original magnification ×50). B, High magnification photograph of the boxed region in A, showing vessels lined by endothelium (c) within the corneal stroma and their association with a large number of inflammatory cells (arrows) (H & E; original magnification ×550).

to the region of injury. That is, the leading edge of the macrophages and neutrophils always precedes the ingrowing vasculature. These observations suggest a two-step mechanism. Macrophages and neutrophils may be attracted to the damaged muscle and upon reaching the ischemic environment within the graft they become activated (17, 19), producing factors capable of stimulating angiogenesis. As regenerating muscle becomes established and the oxygen levels rise in the periphery, the ischemic portions of the muscle could continue to stimulate macrophage and neutrophil chemotaxis and subsequent revascularization. Therefore, the factor released by damaged skeletal muscle may be a chemotactic factor for macrophages and neutrophils rather than an angiogenic factor itself. This is also a potential explanation for the failure of muscles exceeding 3 g to regenerate all of the way to their center. The chemoattractant released by damaged muscles may be stable for a limited time, whereas the centermost part of a large graft can remain necrotic in excess of several weeks. If this part of the muscle loses its chemotactic activity, revascularization and subsequent regeneration would come to a halt and the center of the graft would fill with connective tissue. We tested the muscle extract for chemotactic activity on both endothelial cells and macrophages using a modified Boyden chamber assay (29), but the results were inconclusive. Purification of the extract should give us more exact data.

The angiogenic capacity of damaged skeletal muscle has not been demonstrated previously. In fact, Auerbach *et al.* (30) claimed muscle was not angiogenic on the chick chorioallantoic membrane or in the cornea. Other investigators have demonstrated the ability of endothelial cells to survive injury and form capillaries oriented away from the injury. Faulkner *et al.* (31) explanted muscle fragments into the hamster cheek pouch and using intravital and light microscopy noticed capillaries arising from the explants and anastomosing

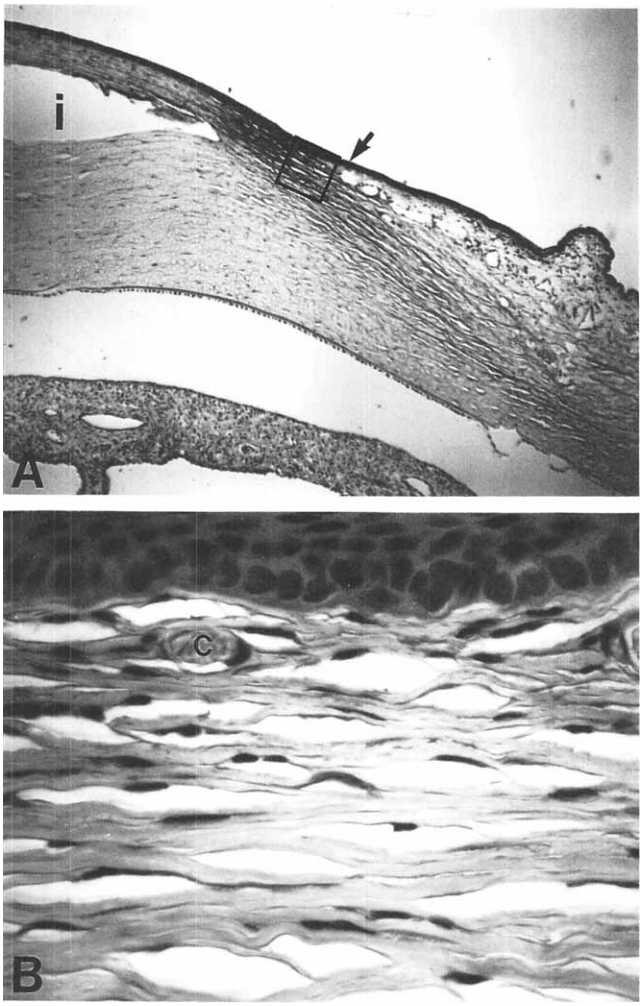

Figure 6. A, Histologic section of a negative angiogenic response to DPBS in Hydron, the control. There is no cellular response to the implant (i) and only a couple small capillary loops extend past the limbus (arrow) into the corneal stroma (original magnification ×50). B, High magnification photograph of the boxed region in A. Notice the lack of inflammatory cells and the presence of a single capillary (H & E; original magnification ×550).

with host vessels. Montesano *et al.* (32) explanted muscle fragments into collagen gels and observed the formation of tube-like structures by endothelial cells growing out of the explants. These studies combined with our own suggest that two mechanisms may be directed toward the same end. Endothelial cells may be stimulated to migrate into damaged muscle and form capillaries. In turn, these vessels could elaborate their own factors, attracting vessels growing away from the graft so an anastomoses can be established.

This study adds an angiogenic factor to the list of growth factors associated with skeletal muscle. Kardami *et al.* (33, 34), Bischoff (8), and Matsuda (35) have demonstrated the presence of skeletal muscle mitogens which promote the proliferation of myogenic cells, the precursors for regenerating muscle. A number of other investigators have reported the existence of muscle-derived factors which enhance survival and neurite outgrowth of both motor and sensory neurons (36–39). Proliferation of myogenic cells, reinnervation, and revascularization are the dominant limiting factors in the regeneration of skeletal muscle. Thus, it appears that skeletal muscle possesses a mechanism to promote its own successful regeneration. A more complete understanding of this mechanism should allow for the enhancement of skeletal muscle regeneration following injury.

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