Chemotaxis in Myogenesis

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Abstract

New skeletal muscle is formed during embryogenesis, and in mature muscle regenerating in response to injury. As part of this process there is a migration of mononucleated muscle precursor cells (myoblasts) to the site of new muscle formation where they fuse into multinucleated new muscle cells. The movement of myoblasts in response to a gradient of soluble factors is the response of chemotaxis. During embryogenesis, the extracellular matrix is important for myoblast adhesion and migration and there is evidence that platelet derived growth factor, possibly produced by the vasculature located ahead of the myoblasts, may provide a chemotactic signal to guide the embryonic myoblasts. During the regeneration of mature skeletal muscle, chemotactic signals seem to be particularly important for attracting myoblasts to the site of new muscle formation. The sequence of chemotactic events is as follows: 1) Soluble factors produced by damaged muscle tissue attract polymorphonuclear leukocytes (PMLs) to the injury site. These factors are produced within 30 minutes of injury, they can persist for several days and their production is prevented by local irradiation of the muscle prior to injury. 2) In turn, soluble factors produced by the PMLs enhance the attraction of macrophages to the injury site. The damaged muscle tissue itself also directly chemoattracts macrophages. 3) Myoblasts are attracted by soluble factors produced by macrophages (but not by PMLs). 4) Myoblasts are also attracted by factors produced directly by the damaged muscle tissue itself. The production of these chemoattractant factors is prevented by irradiation and appears to require an intact vasculature.

Key words: chemotaxis, myoblasts, migration, skeletal muscle, embryogenesis, regeneration, leukocytes, cytokines.

Chemotaxis is the movement of cells in a particular direction in response to a gradient of solubilised attractant, which implies that something must be producing the chemoattractant signal at some distance from the cells of interest. Similar directional cell movement in response to insoluble gradients of molecules bound to the substratum is termed haplotaxis and the distinction is made as the signals may be mediated by distinct transduction mechanisms [4].

Movement of myoblasts in vivo

The formation of new skeletal muscle results from the proliferation of mononucleated skeletal muscle precursor cells (widely referred to as myoblasts) which fuse together to form young multinucleated muscle cells called myotubes that then mature into myofibres. This process requires that the mononucleated myoblasts migrate to the site of fusion where the new muscles is formed. Myoblasts clearly have a good capacity to migrate *in vitro* [55] and also *in vivo* during embryogenesis [reviewed 22]. In mature skeletal muscle, myoblasts are considered to arise from precursor cells located between the external lamina and the sarcolemma of myofibres and termed satellite cells. Evidence that myoblasts/satellite cells can also migrate within mature skeletal muscle tissue *in vivo* is discussed below.

In a classic paper, Lipton and Schultz in 1979, implanted a pellet of cultured myoblasts under the connective tissue fascia of skeletal muscles of growing young (30 day old) rats and of juvenile or old quail [29], and showed extensive dispersion, fusion and movement of the injected cells through the external lamina of the host myofibres into a satellite cell position. While equivalent migration of myoblasts from an implanted cell pellet was reported in both adult and juvenile quail muscles [29], it is recognised that quail muscles have exceptional myogenicity and it is unclear whether they are equivalent to the situation in adult mammalian muscles. Whether similar migration of these

donor myoblasts might also occur in mature muscles of adult rats was not tested (E Schultz, personal communication). In more recent experiments with myoblast transplantation where primary cultures of myoblasts were introduced by direct intramuscular injection into adult rats [43] or regenerating muscles of dystrophic mice [24, 48] it is consistently reported that there was very little movement of donor cells from the injection site and, in most experiments, the injected cultured cells failed to survive [10, 13]. The exception to this is where host mice were immunocompromised, host muscles were irradiated, or donor myogenic cells were derived from immortalised myogenic cell lines and, under these artificial conditions which were used in the majority of myoblast transplantation studies [17], extensive movement of donor cells was often reported. In our laboratory we have demonstrated movement of donor myoblasts into regenerating muscles of dystrophic mdx host myofibres when slices of fresh muscle, rather than cultured myoblasts, were implanted [12]. It is of considerable interest to determine why such differences exist between the migration of cultured and uncultured myoblasts in these various experimental situations and whether chemotactic factors might be involved.

Beyond these experiments with transplanted cells, there is evidence to suggest that myoblasts in mature muscle may migrate *in vivo* towards an injury site, presumably to participate in the formation of new muscle to replace the damaged myofibres. It appears that myoblast migration *in vivo* can occur by myoblasts/satellite cells "hopping out" from beneath the external lamina and into the interstitial space to move towards adjacent damaged myofibres. In other situations satellite cells may remain beneath the external lamina and rapidly migrate down the length of the myofibre to an area of damage on their parent myofibre. These 2 situations might involve different types of potential chemotactic signals. For example, electron microscopic studies on transverse sections of adult rat muscle regenerating after injury by snake-venom, indicated that in "apparently undamaged" myofibres (located in the central core of the muscle and surrounded by a rim of damaged/regenerating myofibres) satellite cells were activated and replicating [26] and were apparently moving out of the parent fibre into the extrafibre space [30]. This raised the possibility that they might be activated and then migrating out through the external lamina into the interstitial space in response to signals produced by nearby damaged myofibres. Similar conclusions were reached from studies with a split autograft model of whole extensor digitorum longus muscle in rats [41] where myotubes were found from 10 days in one half of the graft which had been devitalised by freezing and thawing before reuniting it with the other untreated half of the whole muscle graft. In crush-injured myofibres from young (30 day) rat muscles, the change in longitudinal distribution of labelled satellite cells along the myofibre [50] suggested that there was a rapid (by 15 hours) migration of satellite cells along the length of the

myofibre (presumably down the space between the sarcolemma and external lamina) towards the injury site.

Thus, during myogenesis *in vivo*, in both developing skeletal muscles and in mature muscle regenerating after trauma, there appears to be movement of the mononucleated myoblasts. Information relating to the potential importance of chemotactic signals during myogenesis in these two different situations, and to the nature and source of such signals is presented below.

Migration of myoblasts during embryogenesis

During muscle morphogenesis, myoblasts show directional cell movement but this is not necessarily in response to a chemotactic signal. Instead it appears that this may be largely the result of myoblasts crawling along a gradient of extracellular matrix (ECM) molecules using contact guidance. Fibronectin, possibly produced by fibroblasts may be of particular importance [9] and there is evidence that hyaluronic acid [7], the E8 fragment of laminin-1 in combination with the receptor α7 integrin [11], and other ECM components and their receptors [56] may also regulate this process. Whether cells lying ahead of the crawling myoblasts might break down the ECM to fragments (see below) which are chemotactic to myoblasts is a theoretical possibility, although there seems no evidence to indicate that this does indeed occur during embryogenesis.

The only study that appears to have identified a chemotactic signal for embryonic myoblasts was by Venkatasubramanian and Solursh in 1984 who used Boyden chambers in tissue culture to demonstrate that platelet derived growth factor (PDGF), PDGF-like factors present in horse serum and chick embryo extract were strongly chemotactic for embryonic myoblasts but not for other mesenchymal cells in quail [62]. Fibronectin (50 or 100 μg/ml) and collagen Type I (120 μg/ml) were not chemotactic. There was a dose response to PDGF with a maximum chemotactic response at 5 units/ml and the results suggested that this response was due to a diffusible gradient of PDGF. PDGF is known to be produced by platelets, macrophages and many other cell types. While the source of this PDGF was not tested *in vivo*, they suggested that it might be derived from endothelial cells of the established vasculature and that the myoblasts migrate towards this PDGF source using fibronectin as a substrate. This was based on observations that myogenesis occurs in the developing limb in vascular-rich regions and fibronectin (which mediates adhesion, alignment and migration of myoblasts) accumulates near the vasculature.

Chemotaxis during myogenesis in mature skeletal muscle

In mature skeletal muscle, as indicated above, there is some evidence that satellite cells (myoblasts) show directional movement in response to injury [26, 50]. Tissue culture studies presented below indicate that these migrating myoblasts may be responding to a chemotactic signal produced at the site of muscle damage. The questions that

we seek to clarify are: (i) what cells produce these signals and (ii) what are the chemotactic signals?

We used Boyden chambers (Figure 1) to measure the chemotactic response of myoblasts derived from primary cultures of mature SJL/J mouse muscle or from the myogenic cell line C2C12 [47]. Our data strongly supported the PDGF results of Venkatasubramanian and Solursh [62] with a strong chemotactic response being seen to the AB and BB isoforms of PDGF, although no response was seen to AA- PDGF. A very strong chemotactic response of myoblasts was also seen to leukaemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF) and transforming growth factor-β1.

In addition, these studies showed that exudate macrophages produced factors that were strongly chemotactic to myoblasts, although this was not seen with resident (unstimulated) macrophages, polymorphonuclear leukocytes (PMLs), tissue culture medium or conditioned medium. Since activated exudate macrophages are known to produce a very wide range of enzymes, ECM molecules and growth factors including those listed above [39] it seems likely that macrophages accumulating at the site of injury may, at least in part, be responsible for producing these factors that attract myoblasts to the injury site. It is well established that many of these and other growth factors, including an FGF-like molecule produced by crush-injured muscle [6, 8], also enhance the proliferation of myoblasts *in vitro* [reviewed 18].

To our knowledge no other factors have been shown to be chemoattractants for myoblasts. However, there are many candidates. For example, expression of the secreted glycoprotein osteopontin (which affects cell adhesion and

migration) by subsets of macrophages increases between 1 and 4 days after injury to skeletal muscle and other tissues [36]. There are a huge range of substances (chemokines, growth factors and extracellular matrix proteins) produced and secreted by macrophages and many other cells which are chemotactic for a variety of cell types (see below) and these remain to be tested for myoblast chemoattraction.

Candidate chemoattractants

Chemokines

Growth factors or cytokines which produce a chemotactic response are called chemokines. These chemokines have been shown to induce the directional migration of various cell types including neutrophils, eosinophils, basophils, monocytes, lymphocytes, fibroblasts and cancer cells [5, 59]. The infiltration and directional movement of many of these cells types is an essential part of the general response to tissue damage. The chemokine superfamily consists of 2 distinct subfamilies which can be distinguished by the presence or absence of a single amino acid residue separating 2 adjacent cysteines located at the N-terminus of their amino acid sequences [5, 59]. The α class of chemokines include IL-8/NAP-1, CTAP III/βTG/NAP-2, platelet factor-4, GRO, ENA-78, SDF-1, SDF-2 and IP-10. Many members of this family chemoattract neutrophils (PMLs) and, as discussed in more detail later, the emigration of PMLs from the vasculature is a very important early event in the regeneration of skeletal muscle. The β class of chemokines includes macrophage chemotactic and activating factor (MCAF)/MCP-1, MCP-2, MCP-3, macrophage inflammatory protein-1a (MIP-1α), MIP-1β, RANTES, I-309, C10 and fic. Thus there is a huge range of candidate chemokine molecules which might also have a direct effect on myoblasts.

ECM molecules and their fragments are chemotactic for many cell types

Extracellular matrix proteins are secreted by many cell types and are an integral part of the interstitial connective tissues and of the external lamina (basal lamina/basement membrane) surrounding myofibres. There is strong evidence from many studies of developing muscles and other tissues, particularly the nervous system [25] that fibronectin and laminin [11] are powerful adhesion and migration molecules. However, in developing tissues these adhesion molecules do not appear to provide directional cues; thus they may generally serve primarily as permissive substrates, defining pathways but not providing information about which direction the cells should move.

The situation appears to be different in mature tissues. After tissue damage, proteases such as matrix metalloproteinase-1 (MMP-1) [63] produced by macrophages, fibroblasts, endothelial and other cell types, may rapidly break down ECM proteins into fragments which chemoattract leukocytes (and possibly myoblasts) to the injury site. In addition to the locally cell-derived ECM, fibronectin also circulates in plasma, it associates with fibrin upon activation of the clotting system and is deposited in tissues
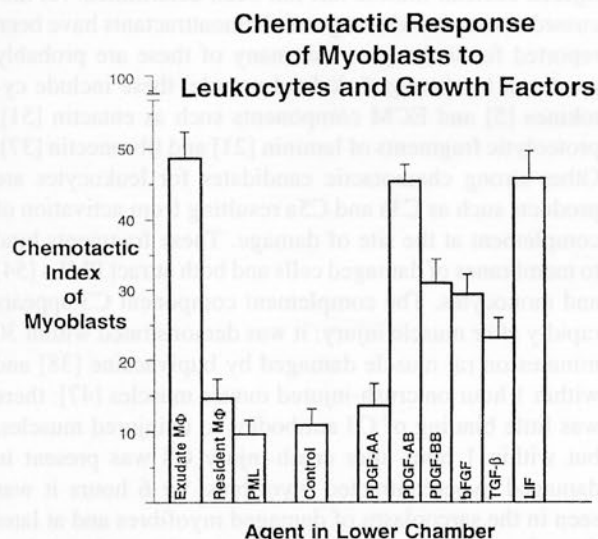

Chemotactic Response of Myoblasts to Leukocytes and Growth Factors

Chemotactic Index of Myoblasts

Agent in Lower Chamber

Figure 1. Summary of the results of experiments using Boyden chambers in vitro to demonstrate the chemotactic response of C2C12 myoblasts (placed in the top chamber) to leukocytes or growth factors (placed in the bottom chamber). These data are based on the paper by Robertson et al (1993).

at sites of wound healing. It has been shown that ECM molecules or their fragments are chemotactic for a range of cells. Both soluble and bound forms of fibronectin, laminin and type IV collagen are chemotactic for human malignant mesothelioma [27] and plasma cell lines [53]. Entactin, which is a major component of basement membranes (such as the external lamina of myofibres), has been shown to be a chemoattractant for PMLs [51]. Other components of intact external (basal) lamina, such as laminin and fibronectin, do not directly affect human PMLs, but selected laminin peptide sequences [21] and fibronectin fragments [37], which may be generated during proteolytic digestion after injury, are chemotactic for PMLs. Furthermore, the interaction of fibronectin fragments with cytokines such as IL-8 can regulate the chemotactic response of neutrophils [19]. It has also been demonstrated that the chemotactic response of fibroblastic cells to fibronectin fragments can be dependent upon the concentration of the fragment and the interaction with other factors [14]. It seems likely that the breakdown of ECM components in the external lamina surrounding myofibres may be responsible, at least in part, for the rapid accumulation of PMLs in injured skeletal muscle. It is important to note that skeletal muscle does not make the $\alpha 1$ chain and laminin-1 which is found in most basal laminae, instead, laminin-2 (merosin) composed of the $\alpha 2$ polypeptide chain is the predominant laminin form in the basal (external) lamina surrounding myofibres [49, 64]. There is no published information to date on the chemotactic effects of laminin -2 or its fragments although this is clearly of central interest with respect to the situation in skeletal muscle.

Source and nature of the factors which are chemotactic for myoblasts

As indicated above, in developing muscle it is suggested that endothelial cells in the vasculature may be the source of PDGF and serve as a chemotactic signal to myoblasts although this speculation has not been tested *in vivo*. Similarly, on the basis of tissue culture studies using Boyden chambers, it is suggested that something produced by the damaged muscle may be a crucial source of chemotactic signals in mature muscle regenerating in response to injury. Leukocytes play a central role in new muscle formation in mature muscle and it is essential to note that leukocytes do not play a similar role in muscle formation during embryogenesis. Thus, the source of the factors, or perhaps the factors themselves, controlling chemotaxis of myoblasts clearly cannot be the same in these two different situations. In the rest of this article we will concentrate upon the situation in mature muscle where new muscle is formed in response to experimental injury or transplantation.

A series of experiments have been carried out in our laboratories over the last 5 years in order to try and ascertain the importance of possible chemotactic signals *in vivo* and the origin of such signals during skeletal muscle regeneration.

Chemoattractants for leukocytes produced by damaged skeletal muscle

After muscle injury, it is well documented that PMLs appear rapidly at the site of damage [47, 60], in rat muscle they were seen within 30 to 60 minutes of injury [38]. Macrophages are the predominant cell from 24 hours after injury. A similar response is seen after any general tissue damage.

Our Boyden chamber studies [47] showed that uninjured muscle had no effect on PMLs whereas muscle removed 3 hours after injury produced diffusible factors that were chemoattractant for PMLs and to a lesser extent for macrophages. This effect was more pronounced in muscle removed at 24 hours after injury. Thus it appears that the leukocytes are actively attracted to the site of muscle damage by some soluble factor that is produced rapidly after injury. That undamaged muscle does not chemoattract leukocytes is also supported by studies in pregnant cows where other tissues were chemoattractant for neutrophils [23]. The venous blood of rabbit skeletal muscles - injured by transient ischaemia for 2-3 hours and reperfusion for 1 hour - contained PMLs with increased chemotaxis and phagocytosis [57]. This was not seen in venous blood from the contralateral limb and confirms that factor(s) rapidly produced by injured skeletal muscle affects PMLs [57]. This effect was not seen after only 1 hour of ischaemia [58] and neither was a chemotactic response on PMLs elicited by crushed muscle removed immediately after injury in our studies [47] indicating that the "chemotactic factor for PMLs" produced after skeletal muscle injury takes some time to accumulate or be produced.

The nature of the chemoattractant(s) produced by the injured skeletal muscle has not been determined. As discussed above, a wide range of chemoattractants have been reported for leukocytes and many of these are probably produced by damaged skeletal muscle: these include cytokines [5] and ECM components such as entactin [51], proteolytic fragments of laminin [21] and fibronectin [37]. Other strong chemotactic candidates for leukocytes are products such as C3a and C5a resulting from activation of complement at the site of damage. These fragments bind to membranes of damaged cells and both attract PMLs [54] and monocytes. The complement component C3 appears rapidly after muscle injury: it was demonstrated within 30 minutes on rat muscle damaged by bupivacaine [38] and within 1 hour on crush-injured mouse muscles [47]: there was little binding of C3 antibodies to uninjured muscles, but within 1 hour after crush-injury C3 was present in damaged hypercontracted myofibres, by 6 hours it was seen in the sarcoplasm of damaged myofibres and at later times in necrotic tissue where it appeared to peak at 3 days [Robertson TA, unpublished observations]. In these studies no binding was seen with antibodies to C9. The rapid accumulation of mitochondria at the ends of damaged myofibres [40] may contribute to complement activation [16] at these sites.

In summary, possible sources of the chemoattractant(s) for PMLs and macrophages produced by damaged skeletal muscle tissue are:
1. Direct damage to resident cells in the muscle tissue itself resulting in the release or increased expression of various factors - the candidate cell types affected include:
- blood vessel cells - endothelial, smooth muscle cells or pericytes;
- nerves;
- interstitial cells - macrophages [33, 42], dendritic cells [42], fibroblasts, mast cells;
- the myofibres - component myofibrillary proteins, mitochondria or other cell organelles, membranes, heat shock proteins etc. Changes in the electrical activity of the plasmalemmal membrane;
2. Proteolytic breakdown of ECM components in the external lamina around myofibres or in the interstitial connective tissue between the myofibres;
3. Vascular response to injury - clotting and the accumulation of platelets, the complement cascade, other systemic factors.

Ultrastructural studies of adult mouse muscle damaged by chemical injury (a small transverse zone of the exposed surface was painted with Karnovsky's fixative - a mixture of paraformaldehyde and glutaraldehyde - which preserves the tissue structure) revealed rows of PMLs lined up beneath the external lamina of apparently undamaged myofibres adjacent to damaged myofibres (Figure 2) within 12 hours [Robertson TA, unpublished observations]. This suggests that the PMLs had been stimulated to move through the external lamina onto the sarcolemmal surface of the myofibre, in response to some factor produced by the myofibre itself. What might such a factor be?

Other information is gleaned tangentially from experiments with whole grafts of intact extensor digitorum longus (EDL) muscles in mice [44, 45]. In this situation, the vascular and neural supply of the orthotransplanted EDL is completely disrupted and the entire muscle is enclosed in a connective tissue sheath. The muscle undergoes necrosis followed by complete regeneration which is dependent upon the zonal infiltration of leukocytes closely associated with revascularisation of the graft. The first infiltrating PMLs and macrophages are seen at the circumference of the necrotic grafts at 2 to 3 days after transplantation. This leads us to ask what is the factor associated with the necrotic muscle that is clearly still present at 2 days and attracts the leukocytes to the graft?

It is well documented that PMLs produce many diffusible factors that actively attract macrophages to an injury site and that these leukocytes in turn produce factors that attract

Figure 2. An electron micrograph of polymorphonuclear leukocytes (P) located beneath the external lamina (arrows) of an "apparently normal" adult muscle fibres at 12 hours after chemical injury with Karnovsky's fixative. Note the myonucleus in the sarcoplasm of the myofibre (Bar = 1 μm). Inset: a light micrograph of the same region. A necrotic myofibre resulting from the direct injury was located one myofibre away (not shown). Numerous polymorphonuclear leukocytes can be seen in close alignment with the myofibre. Note the same myonucleus as depicted in the electron micrograph (arrowhead) (Bar = 10 μm).

additional macrophages. The role of circulating leukocytes is examined further in this paper.

In particular, we tested whether the damaged muscle itself (i.e. in the absence of infiltrating leukocytes) produces diffusible factors that attract macrophages to the injury site.

In these experiments, leukocyte-depleted mice were produced by whole body irradiation (WBI) 2 days prior to injury.

Chemoattractants for myoblasts

As indicated above, the macrophages, but not the PMLs, produce factors that attract myoblasts to the injury site. We do not know whether the damaged muscle itself (i.e. in the absence of infiltrating leukocytes) produces diffusible factors that attract myoblasts to the injury site. We therefore carried out experiments *in vitro* using Boyden chambers with damaged muscles removed 2 or 3 days after crush-injury being placed in the bottom chamber, and tested against myoblasts in the top chamber (Figure 3). To eliminate bone-marrow derived leukocytes from regenerating muscle, the body of the host mouse was irradiated 2 days prior to injury, with only one leg being protected from irradiation (Figure 4). Additional studies were carried out *in vivo* to observe directly the number and location of myoblasts in crush-injured muscles of leukocyte depleted (WBI) host mice, by using desmin antibodies to directly identify the myoblasts in longitudinal sections of regenerating muscles.

Strain-specific differences in the chemotactic response

There are marked strain-specific differences in muscle regeneration between SJL/J and BALB/c mice, with SJL/J mice having superior new muscle formation after crush injury which is associated with twice the number of leukocytes in regenerating lesions at 3 days [34]. Possible strain-specific differences in the chemoattractant for leukocytes produced by damaged skeletal muscles from these 2 strains have been investigated in other studies and the results are briefly presented here at the end of the Experimental Section. One of these studies involved cross-transplantation of whole muscle grafts between these 2 strains (Roberts P, McGeachie JK, Grounds MD. Manuscript in preparation): whole muscle grafts undergo necrosis in a situation where the vascular and neural supply is completely disrupted and regeneration depends on the zonal infiltration of leukocytes starting at the periphery of the graft [44]. Another study compared the chemotactic effect of crush-injured muscles removed from these 2 strains of mice using Boyden chambers *in vitro* (Mitchell CM, McGeachie JK, Grounds MD, unpublished observation).
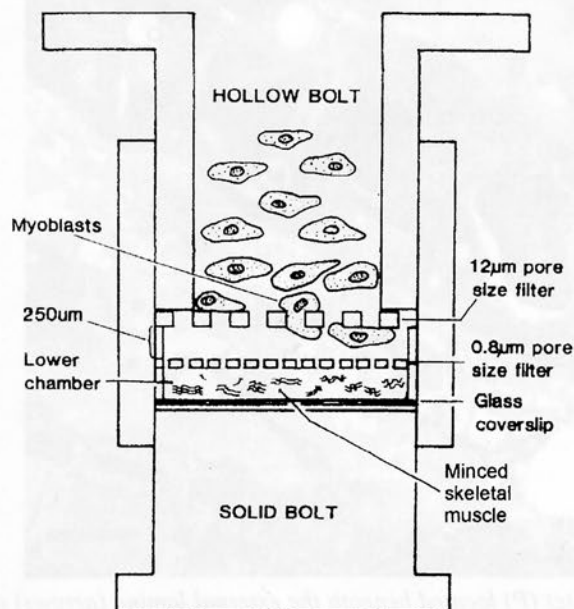

MODIFIED BOYDEN CHAMBER

Figure 3. Diagram of the modified Boyden chambers used in the in vitro experiments. This arrangement shows the filter sizes used to measure the chemotactic response of myoblasts.
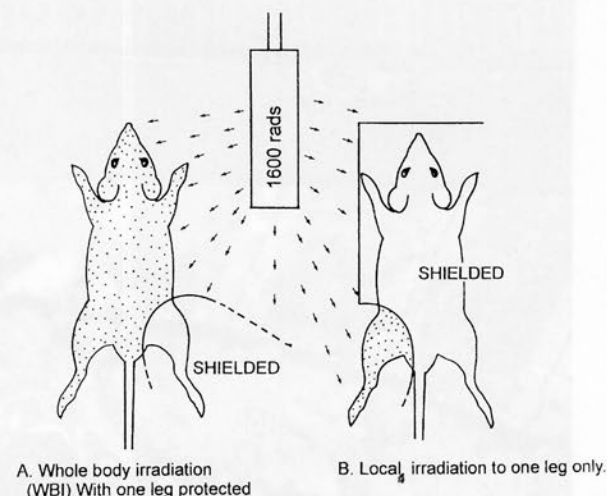

Figure 4. Diagram of the irradiation procedures on mice. A. Whole body irradiation (WBI) with one leg protected. WBI eliminates all of the circulating bone-marrow derived leukocytes prior to injury; local cell replication is unaffected in the one leg (right) that is protected from the irradiation source. B. Local irradiation to one leg only. Local irradiation prevents replication of all resident cells in the muscle tissue (in response to injury). Mice were irradiated 2 days prior to crush injury to TA muscles in both legs. Muscles were sampled within 3 days of the crush injury.

Methods

Animal procedures

Inbred female specific pathogen free SJL/J mice aged 6-8 weeks were used in experiments unless otherwise indicated. All animal procedures were carried out in strict accordance with the guidelines of the National Health and Medical Research Council of Australia. Injured muscle resulted from a transverse crush injury to the tibialis anterior (TA) muscles in the hind legs as described in detail by Mitchell et al [34]. Grafts of whole intact extensor digitorum longus (EDL) muscle were transplanted orthotopically as described in detail elsewhere [44, 45]. Whole body irradiation with 1600 rads, either with or without one leg shielded by lead sheeting (2 mm thick), or local muscle irradiation to only one leg of other mice (Figure 4), was carried out on animals anaesthetised by intraperitoneal injection of Nembutal (30 µg/gm body weight) as described by Robertson et al [46].

Boyden chamber experiments

For the tissue culture studies of chemotaxis, a Boyden chamber was set up as illustrated in Figure 3. The 2 chambers were separated by a sandwich of 2 polycarbonate nucleopore filters (Costar No 110409, USA), one of a 0.8 µm pore size placed immediately above the muscle and another of 0.5 µm or 12 µm pore size placed on top. A 5 µm pore size was used in experiments where macrophages were placed in the top chamber because of the small size of macrophages (13-20 µm). The 12 µm pore size filter (coated with fibronectin before use) was used in experiments where myoblasts were placed in the top chamber because of the diameter of the C2C12 myoblasts (15-40 µm).

Exudate peritoneal macrophages (0.8 ml of 10^6 cells/ml) used in the top chamber were obtained by harvesting peritoneal macrophages 48 hours after injection of 0.5 ml of sodium thioglycollate broth (Difco) into the peritoneal cavity of female SJL/J mice (to stimulate cells from the blood) [47]. At 48 hours post-injection, the exudate consists of 80% macrophages and 20% polymorphonuclear leukocytes. In experiments where myoblasts were placed in the top chamber, 0.8 ml of approximately 0.25 x 10^6 C2C12 cells/ml were used. A sample of muscle (approximately 0.1 gm) was placed in the lower chamber with no additional fluid (either 1 whole crushed TA muscle was minced, or 2 EDL grafts were pooled and minced together). Controls had 100 µl of Dulbecco's modified Eagle's medium in the bottom chamber.

The Boyden chambers were incubated for 2 hours in a CO_2 incubator at 37°C. The filter was removed, fixed in 10% buffered formal saline, inverted so that the 'lower surface' was uppermost (the surface where migrated cells would be attached), stained with haematoxylin and eosin and mounted in DePeX medium (Gurr) for light microscope examination. The filters were blind-coded and the average number of cells on the 'lower surface' of each filter

(in fields viewed with a x 25 microscope lens), was scored as: - (no cells), ⌠ (occasional, 1-2 cells), + (3-5 cells), ++ (6-9) or +++ many, ⎮ 10 cells).

Desmin immunohistochemistry

Myoblasts were identified in tissue sections by staining with desmin antibodies using an antigen retrieval method on muscles fixed for 8 hours in 4% paraformaldehyde and processed through paraffin wax. Antigen retrieval was carried out on 8 µm longitudinal sections in 10 mmol/l citrate buffer (pH 6.0) in a microwave using a method modified from Shi et al 1991 [52] (This methodology is described in detail in Lawson-Smith M, McGeachie JK, Davies M , Maley MAM, Grounds MD, manuscript in preparation). After antigen retrieval, desmin was detected using a polyclonal rabbit antibody (1:200) prepared against chicken gizzard smooth muscle (Biogenex Lab), followed by biotinylated anti-rabbit antibody (1:200) raised in donkeys (Jackson Immunoresearch laboratories), avidin D peroxidase (1:200) (Vector Laboratories) and diaminobenzidine (DAB)/metal substrate (Pierce) to produce a brown precipitate. Nuclei in sections were counterstained blue with haematoxylin.

Experiments

Chemotactic response of macrophages to damaged muscle in vitro

SJL/J mice were subjected to whole body irradiation with the right leg shielded by lead from the irradiation source. At 48 hours after irradiation, when the numbers of blood-borne leukocytes are undetectable [46], the TA muscles of both legs were crush injured. Blood was collected from the tail tip at the time of surgery and blood smears examined to confirm the absence of leukocytes. Control mice (with circulating leukocytes) had the body and right leg shielded and only the left leg exposed to the irradiation source, and all were similarly crush injured after 48 hours. Other controls, were muscles with and without crush injury from SJL/J mice with no irradiation, and also muscles from 10 week old dystrophic mdx mice, which have inherent muscle necrosis and regeneration [32]. At 2 days after injury (4 days after irradiation), TA muscles from the right (protected) and left (irradiated) legs were sampled, minced and placed in the lower chamber of individual Boyden chambers. Muscle from one leg was placed in each chamber and 2-4 chambers used for each experimental group. Exudate macrophages from peritoneal washings were placed in the top chamber for chemotactic analysis.

Chemotactic response of myoblasts to injured muscle

TA muscles of mice (either non-irradiated or irradiated as above), crush injured at 3 or 2 days prior to sampling, were placed in the lower Boyden chamber and tested against myoblasts of the C2C12 myogenic cell line placed in the top chamber for chemotactic analysis in vitro. In one experiment, whole EDL muscle grafts removed at 3 days after transplantation, were dissected free of the underlying

TA, and the paired muscles from one mouse were minced (to produce an equivalent amount of tissue to that used for the crushed muscle experiments), placed in the lower Boyden chamber and tested against C2C12 myoblasts.

For *in vivo* observation of a possible "chemotactic response of myoblasts towards a site of muscle injury in the absence of infiltrating leukocytes", 5 SJL/J mice received local irradiation of the left leg (2 mice) or WBI with one leg protected (3 mice) prior to crush injury as described above. Whole TA muscles were sampled at 3 days after injury, processed through paraffin wax and longitudinal tissue sections stained with antibodies to desmin (in order to visualise myoblasts). Muscles were sampled at 3 days, as earlier experiments (unpublished) have shown that relatively few desmin positive myoblasts can be identified before this time.

Results and Discussion

QUESTION 1: Does the damaged skeletal muscle itself, in the absence of infiltrating leukocytes, produce chemotactic factors that attract macrophages? The answer is YES.

The Boyden chamber experiments (Table 1) confirmed that muscle injury produces soluble chemoattractants for macrophages. The dystrophic mdx muscle, which has inherent foci of necrotic and regenerating myofibres, also elicited a strong chemotactic response. Local irradiation of the muscle 2 days prior to crush-injury almost eliminated the production of these soluble factors by the damaged muscle (Table 1). This unexpected effect of local irradiation is difficult to explain. Irradiation disrupts DNA replication and hence cell proliferation [46]. It is known that the PML chemoattractant is normally produced very rapidly, within hours of damage, well before replication can occur, and presumably results from the release, breakdown or synthesis of some local factor in the damaged muscle. It is usually considered that irradiation does not destroy enzymatic activity and the general transcription and translation of genes into proteins. However, the very high dose of irradiation (1600 rads) administered 2 days prior to muscle injury appears to have completely disrupted the critical cellular function responsible for production of the soluble chemoattractant.

In mice where circulating leukocytes had been eliminated by WBI, there was no chemattractant effect of the irradiated crushed muscle on macrophages, corresponding with the results of local muscle irradiation only. In the protected leg of the same mice, the chemoattractant for macrophages produced by crush injury appeared unaffected by WBI. This indicates that the circulating leukocytes which normally accumulate at the site of muscle damage are alone not responsible for attracting macrophages to the injury site. The results argue strongly for the production of a chemoattractant factor(s) for macrophages being produced directly by resident cells or other local components of the damaged muscle itself.

QUESTION 2: Does the damaged skeletal muscle itself, in the absence of infiltrating leukocytes, produce chemotactic factors that attract myoblasts? The answer is YES.

Crushed muscles

The Boyden chamber experiments showed that C2C12 myoblasts were chemoattracted to soluble factors produced by skeletal muscle at 3 days after crush injury (Table 2). This chemotactic response of myoblasts to injured muscle has not been demonstrated previously. Muscle removed at 2 days elicited a greater response than muscles removed at 3 days. With muscles removed at 1 day after injury, some movement of myoblasts onto the underside of the filter was seen and it was observed that many myoblasts on the top surface of the filter were crowded around the pores as though they were tempted to crawl through. This strongly suggested that a weak signal was being detected by the myoblasts. Such an accumulation of myoblasts around the pores on the top surface of the filter was never

Table 1. Chemotactic response of macrophages to skeletal muscle. Exudate peritoneal macrophages were placed in the top compartment of a Boyden chamber and TA muscles removed 2 days after crush injury were placed in the bottom compartment (Figure 1). The muscles were removed from normal SJL/J mice, from mice where only the right leg was subjected to irradiation and from mice which had received whole body irradiation (WBI) with one leg protected prior to crush injury. Note: Irradiation prevents replication of local cells and WBI removes leukocytes from the circulation. In addition, muscles were taken from dystrophic mdx mice and uninjured SJL/J mice. The number of macrophages on the underside of the filter is scored from +++ (many) to - (none).

non-irradiated SJL/J Crushed	left leg only irradiated. Crushed		WBI right leg protected. Crushed		dystrophic mdx	control SJL/J Uncrushed	control DMEM
	left	right	left	right			
+++	++		-	+++	+++	+	-
++	++		-	++	+++	+	-
+++				+++	++	+	-
++				+++	+++	+	-

Table 2. *Chemotactic response of myoblasts to skeletal muscle. C2C12 myoblasts were placed in the top compartment of a Boyden chamber and TA muscles removed at 1-3 days after crush injury were placed in the bottom compartment (Figure 1). The muscles were removed from normal SJL/J mic at 1, 2 or 3 days, and thereafter at 3 days from mice where only the right leg was subjected to irradiation and from mice which had received whole body irradiation (WBI) with one leg protected prior to crush injury. Note: Irradiation prevents replication of local cells and WBI removes leukocytes from the circulation. As a control, muscles were taken from uninjured SJL/J mice. Data from EDL grafts sampled at 3 days are also included. The number of myoblasts on the underside of the filter is scored from +++ (many) to - (none).*

non-irradiated				WBI right leg protected. Crushed		Control Uncrushed	Control DMEM
Crushed			EDL				
day 1	day 2	day 3	day 3	left	right		
	+++	++	-		++	+	-
+	+++	++		-	++	-	-
+	++	++			++	+	-
	+++	++		-	++	-	-

noted in controls such as MEM or uninjured muscle. Since our earlier studies have already shown that macrophages produce soluble factors that chemoattract myoblasts it seemed likely that the myoblasts were responding to the presence of these inflammatory cells. Whether damaged muscle itself (in the absence of infiltrating inflammatory cells) produces signals which are chemotactic for myoblasts, was tested *in vitro* using injured muscle from mice which had received WBI with one leg protected 2 days prior to crush injury. The results showed that there was a strong chemotactic response elicited by the leg which had been protected from irradiation, demonstrating that soluble factors produced by the damaged muscle itself, in the absence of inflammatory cells, were strongly chemotactic for myoblasts. Irradiation of the muscle ablated this chemotactic signal.

The chemotactic response of myoblasts in such irradiated mice was also assessed in tissue sections stained with desmin (Figure 5). In normal SJL/J mice, an extensive zone of inflammatory cells was present with removal of much of the necrotic damaged tissue, corresponding to earlier descriptions of crushed muscle after 3 days [34]. Desmin staining revealed many myoblasts throughout this regenerating zone with many concentrated at the tips of damaged myofibres near the central necrotic zone [Figure 5 a, b]. Muscles which had been irradiated prior to injury showed weak desmin staining of cells with little cytoplasm throughout the regenerative muscle, these cells were few in number presumably because proliferation had been prevented by the irradiation. In WBI mice (where circulating leukocytes had been eliminated), there was no zone of inflammatory cells and no removal of the necrotic tissue, although some breakdown of the sarcoplasm of damaged myofibres was apparent (Figure 5 c-f). In the irradiated leg of these mice, weakly staining desmin-positive cells were seen scattered throughout the muscle (Figure 5d). In the leg

of these WBI mice which had been protected from irradiation, strongly staining desmin positive cells were present and in several instances they were seen concentrated at the edge of the necrotic zone within the ends of longitudinal sections of "relatively undamaged segments" of the injured myofibres (Figure 5 c, e, f). Such concentrations of myoblasts were not apparent in other areas of the TA muscle away from the injury site. This result strongly supports the idea that the myoblasts had migrated down the length of the myofibres from regions distant to the injured segment in response to factors produced by the damaged tissue itself (this is clearly independent of any chemotactic effect of infiltrating macrophages as they were not present in this experiment).

Candidate chemotactic signals

In situ examination of injured muscles is necessary to reveal potential changes in amounts of candidate chemoattractants and to identify the cells responsible for producing them. Such studies carried out with riboprobes [20] or antibodies to bFGF [2] in regenerating adult muscle show a marked increase in bFGF within the damaged myofibres, in inflammatory cells and in myoblasts [15]. Time course studies of crush-injured muscles reveal that by 1 hour there was no detectable change in bFGF staining, at 3 hours increased amounts of bFGF were localised at the ends of sealed stumps of damaged myofibres and bFGF increased in damaged myofibres from this time [3]. This pattern of expression of bFGF is compatible with a role for both damaged myofibres and leukocytes in producing such a chemoattractant for myoblasts (and for leukocytes).

An increase in LIF and IL-6 mRNA was also demonstrated by 3 hours in crush injured mouse muscle [28]. Levels of LIF were higher than IL-6 and peaked at 24 hours, 1 day earlier than IL-6. The time course of this *in vivo* increase, combined with the potent chemotactic effect of LIF on myoblasts demonstrated in Boyden chambers

[47] indicates that LIF might be a significant chemoattractant for myoblasts *in vivo*. The source of this rapid increase was not determined although LIF is known to be produced by many mononucleated cells as well as myoblasts and, perhaps to some extent, by regenerating myofibres. Further information on the possible role of LIF comes from polymerase chain reaction amplification of mRNA for LIF and IL-6 mRNA [28] in crushed muscles from mice where one leg was irradiated or mice received WBI with one leg protected prior to injury (as in the present experiments).

This was a collaborative study between our laboratory and Dr L Austin and J Kurek in Melbourne in 1993 [unpublished observations]. At 3 days after crush injury, a marked increase in LIF and IL-6 mRNA was seen compared to levels in uninjured muscles. This increase was unaffected by any of the irradiation treatments. Since this increase after injury is not ablated by irradiation, this indicates that neither of these factors play a central role as chemoattractants *in vivo*.

Members of the TGF-β family are also potent chemoattractants for myoblasts and inflammatory cells and studies with antibodies to these growth factors have been carried out on segmentally injured muscles [McLennan IS, personal communication; manuscript under revision]. The results showed a rapid increase in TGF-β2 immunoreactivity within 5 hours after injury and this accumulated in the damaged myofibre at the junction between the intact and necrotic portion of the myofibre.

While the precise source and nature of the chemoattractant(s) is not yet known, the relative importance of candidate molecules can be tested by comparing their levels in
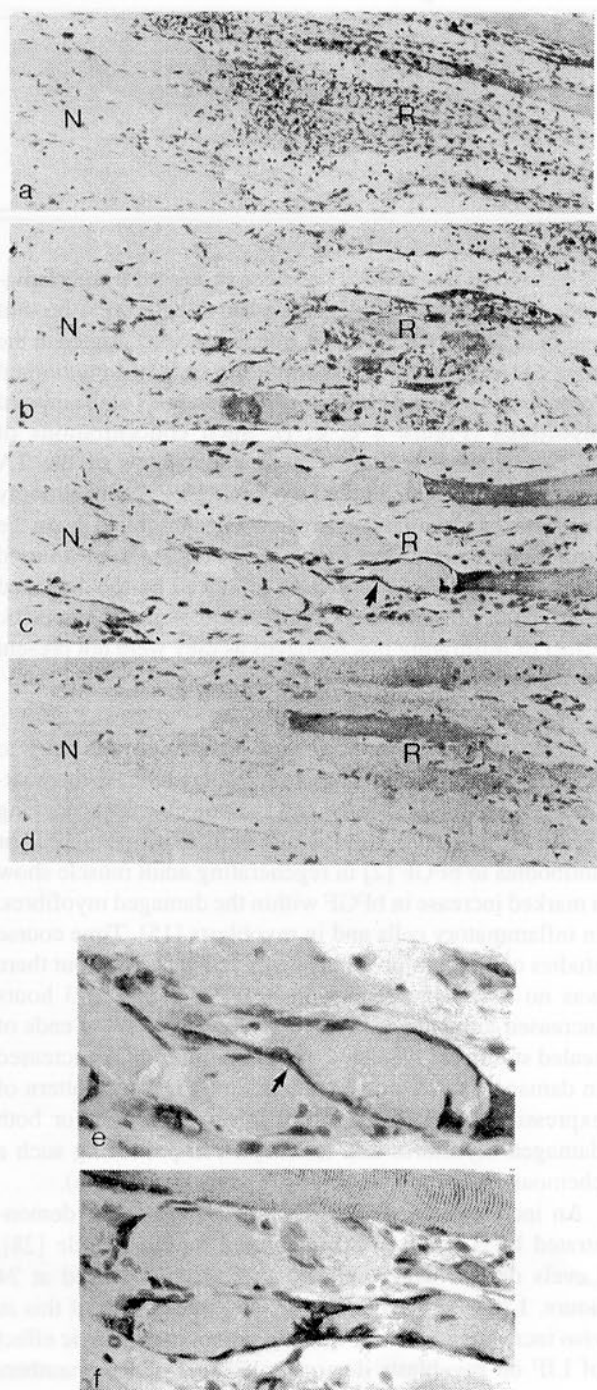
Figure 5. Longitudinal sections of muscles removed at 3 days after crush injury and stained with desmin antibody. Similar fields are shown at the junction of the remaining necrotic muscle [N] resulting from the direct injury (on the left hand side) and the adjacent regenerating muscle zone [R]. The brown peroxidase reaction product identifying the bound desmin antibody unfortunately cannot be distinguished from the blue nuclear counterstain in the black and white photographs. Magnification of the photographs is x 160 apart from e and f which are x 400. a, b. In non-irradiated muscles (from 2 different mice) many inflammatory cells are present and some of the necrotic muscle has already been removed by phagocytosis. At the junction of the necrotic muscle and the zone of inflammatory cells, many darkly staining nuclei line the contours of the damaged myofibres; these are the desmin-positive myoblasts c-f Crush injured muscle from mice subjected to whole body irradiation 2 days prior to injury: note the lack of inflammatory cells in c and d. In the protected (right) leg, c, desmin stained myoblasts were conspicuous within the ends of damaged myofibres at the edge of the necrotic muscle zone. Further away from this area (off to the right hand side) relatively few desmin positive cells were seen. High powered views of desmin positive myoblasts within the end of a damaged myofibres (arrow) near the necrotic muscle, and a similar zone from the protected leg of a second mouse, are shown in e and f respectively. d. In the irradiated (left) leg, desmin positive cells were not noted at the edge of the necrotic muscle zone, although pale staining desmin positive myoblasts were distributed throughout the adjacent muscle (not shown).

normal crush-injured skeletal muscle with muscles that have been irradiated prior to crush injury (as production of the soluble chemoattractants is prevented by such irradiation). In addition, whole muscle grafts at 1 and 3 days after transplantation elicited no chemotaxis by myoblasts (see below) and therefore it would be expected that levels of candidate chemoattractants would also be low or non-existent in this model, compared with crush-injured muscles.

Whole muscle grafts

Examination of whole muscle grafts offered an alternative approach to examining the chemotactic signals produced by damaged muscle in the absence of infiltrating leukocytes. Whole muscle autografts of EDL muscles were removed at 3 days, placed in the lower compartment of a Boyden chamber and tested against C2C12 myoblasts (Table 2). No migration of the myoblasts was seen. An identical result was seen with EDL grafts removed at only 1 day after transplantation. This result strongly suggests that connection with the vascular supply (or an intact nerve supply) is necessary to generate the chemotactic signal for myoblasts. (Although it was not tested, it would be expected that such EDL grafts would produce chemotactic signals for leukocytes at 3 days as emigration of these cells is seen *in vivo* at the periphery of grafts at this time.)

Strain specific differences in chemotaxis

The capacity for regenerating muscle to chemoattract leukocytes was compared between 2 strains of mice using whole muscle grafts [Roberts P, McGeachie JK, Grounds MD; manuscript in preparation]. In autotransplants of whole EDL muscles, earlier revascularisation, infiltration of PMLs and macrophages, and activation of myoblasts were consistently seen in at 2 and 3 days in SJL/J mice in comparison with the BALB/c mice. However, when BALB/c muscles were implanted into the SJL/J hosts, the regenerating muscles had a similar histological appearance to that of the SJL/J autografts at 3 days. This lack of difference between 2 strains of grafts implanted into the SJL/J hosts indicated that it is not the muscle itself that is determining the timing of the regenerative process, and it appears that there is little difference between muscle from the 2 strains in their chemotactic capacity to attract leukocytes in this situation. The reverse cross-transplantation experiments using BALB/c hosts confirmed that the pattern of regeneration was determined by the host environment. *These cross-transplantation experiments indicate that it is the avidity of the PMLs and/or macrophages in the SJL/J host mice that results in the more rapid cellular activity in EDL grafts transplanted into this strain of mice.* This critical role of the host environment in determining the timing and efficiency of muscle repair between strains is reminiscent of the differences in muscle repair seen between old and young animals [31].

The conclusion that leukocytes from SJL/J mice have a more vigorous chemotactic response than those from BALB/c mice is supported by Boyden chamber studies using combinations of crushed muscles and exudate
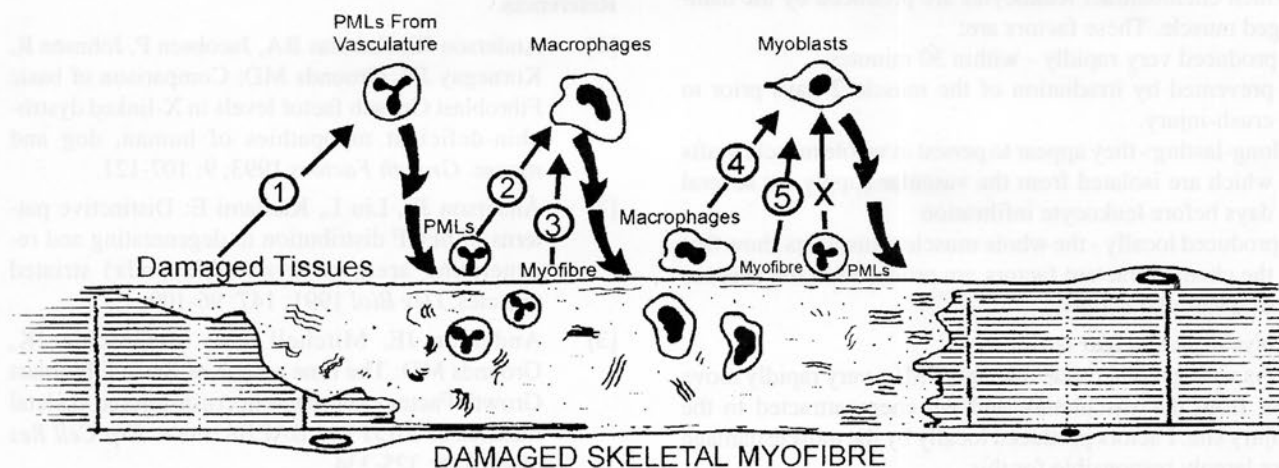
CHEMOTACTIC PATHWAYS

Figure 6. Summary of probable chemotactic pathways during skeletal muscle regeneration in response to crush injury. Polymorphonuclear leukocytes (PMLs) are chemoattracted very rapidly to the injury site by soluble factors produced by the damaged tissue (1). The macrophages respond to soluble factors produced by the PMLs (2) and also to factors produced directly by the damaged skeletal muscle itself - this might be from damaged myofibres, muscle nuclei or other resident cells (3). The myoblasts are attracted to factors produced by macrophages (but not PMLs) (4) and the damaged muscle tissue itself (5). Likely chemotactic signals are C3, chemokines, growth factors like bFGF and ECM fragments (see text).

macrophages from these 2 strains of mice [C. Mitchell C, McGeachie JK, Grounds MD, unpublished observations]. This difference in leukocyte chemotactic response is probably regulated by some factor in the host environment, as whole body irradiation/bone marrow replacement studies show that the macrophage activity is not dependent upon the genotype of the bone-marrow derived cells [35]. In confirmation of the results of Robertson et al [47] crush-injured muscle from both strains removed after 3 days was significantly ($p < 0.0001$) more chemoattractant for exudate macrophages than was uninjured muscles, with the chemotactic index (CI) being 2-3 fold higher (mean CI of 0.291 and 0.068 respectively) [Mitchell et al., unpublished observations]. The crush-injured SJL/J muscle was slightly but significantly ($p < 0.05$) more chemattractive for SJL/J macrophages than the equivalent injured BALB/c muscle (mean CI of 0.291 and 0.251 respectively). This slight difference in the *in vitro* chemotactic capacity of crushed muscle from the 2 strains, contrasts with the *in vivo* situation with whole muscle grafts (above),. This difference can be attributed to the larger number of leukocytes in crushed SJL/J muscles sampled for the Boyden chamber studies, as it is known that twice the number of inflammatory cells are present in crushed SJLJ/J compared with BALB/c muscle at 3 days [34]. In contrast, there is no immediate infiltration of leukocytes into whole muscle grafts as the vascular supply is completely disrupted at the time of transplantation.

Conclusions (see Figure 6)

Leukocytes

1. After injury to mature skeletal muscle, soluble factors which chemoattract leukocytes are produced by the damaged muscle. These factors are:
- produced very rapidly - within 30 minutes.
- prevented by irradiation of the muscle 2 days prior to crush-injury.
- long-lasting - they appear to persist in whole muscle grafts which are isolated from the vascular supply for several days before leukocyte infiltration
- produced locally - the whole muscle transplants show that the chemoattractant factors are produced in the absence of a vascular supply.

Polymorphonuclear leukocytes

As with general tissue damage, PMLs very rapidly move out from the vasculature and are chemoattracted to the injury site. Factors produced locally by the muscle damage are largely responsible for this.

Macrophages

1. Macrophages are probably chemoattracted by factors produced by the PMLs at the damage site.
2. Macrophages are also strongly chemoattracted by factors produced directly by the damaged skeletal muscle itself (in the absence of infiltrating leukocytes): this might involve myofibres, cells resident in the interstitial connective tissue, or serum derived factors.

Myoblasts

1. The factors responsible for attracting myoblasts to sites of muscle damage are:
- produced rapidly: they are present by day 1, highest at day 2 and still pronounced at day 3 after crush injury
- prevented by irradiation.
- dependent on an intact vasculature (or intact innervation) as they were not detected in whole muscle grafts tested at 1 or 3 days after transplantation
2. Myoblasts are chemoattracted to factors produced by activated macrophages (but not by PMLs).
3. Myoblasts are also attracted directly by soluble factors produced by the necrotic/damaged muscle itself (in the absence of infiltrating macrophages).

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