Accumulation of PDGF\(^+\) cells and internalisation of the PDGF receptor at myotendinous junction following modified hindlimb muscle use in the rat

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ABSTRACT

Morphological observations have shown previously that myotendinous junctions (MTJs) are sites where the associations between the cytoskeleton and the cell membrane are extensively remodelled during muscle growth and modified mechanical loading. The platelet derived growth factor (PDGF) molecule has been shown to induce cytoskeletal remodelling at focal contact sites of myoblasts in culture, the analogous structures of MTJs. The goals of the study were to determine whether PDGF is synthesised by mononuclear cells and whether PDGF receptors are internalised at the MTJs of the soleus muscle experiencing reloading. We also examined whether ED2\(^+\) macrophages that are nonphagocytic and activated inflammatory cells at MTJs during reloading secrete PDGF. Results obtained by immunohistochemistry showed that there was an increase in the number of cells expressing PDGF at remodelling MTJs and that the ED2\(^+\) macrophage population does not express PDGF at MTJs. According to morphological criteria, fibroblasts would be the logical candidates to secrete PDGF molecules near MTJs. Furthermore, the modification in muscle loading resulted in internalisation of PDGF receptors concentrated at the MTJ which accumulated predominantly around muscle nuclei. The enrichment of PDGF receptors and PDGF\(^+\) cells at MTJs and the internalisation of PDGF receptors during remodelling of MTJs suggest that PDGF may influence remodelling of MTJs following modified muscle use.

Key words: Skeletal muscle; mechanical reloading; cytoskeletal remodelling; cytokines; ED2\(^+\) macrophages.

INTRODUCTION

Myotendinous junctions (MTJs) are regions of muscle cell membranes where tension generated by myofibrils is transmitted to the extracellular matrix (ECM) by a series of proteins that mediate actin-membrane interactions (for review, see Tidball, 1991). Microscopic observations have shown that macrophages from different phenotypes are present at MTJs during modified muscle use (St Pierre & Tidball, 1994\(a\)). However, the ED2\(^+\) macrophages, nonphagocytic and resident inflammatory cells in muscle and other tissues, are the only subset of macrophages known to increase in number and size near MTJs following 2 d of reloading (St Pierre & Tidball, 1994\(a\)). These observations suggest that ED2\(^+\) macrophages through their secretory products may play an important role at MTJs.

Although macrophages can secrete many factors that may feasibly affect MTJ structure (Nathan, 1987), receptors for platelet-derived growth factor (PDGF) are the only growth factor receptors known to be enriched at MTJs of fully-differentiated muscle fibres (Tidball & Spencer, 1993). PDGF receptors are transmembrane molecules that autophosphorylate and phosphorylate other proteins on tyrosine following PDGF binding (Ek & Heldin, 1984). The presence of PDGF receptors at MTJs suggests that these tyrosine kinases may play a regulatory role in MTJ remodelling. This possibility is supported by several studies that have shown in vitro that PDGF stimulation of fibroblasts and skeletal muscle cells induces reorganisation of the cytoskeleton and loss of vinculin from focal contacts (Herman & Pledger, 1985; Herman et al. 1986; Tidball & Spencer, 1993). Other findings have also demonstrated that ECM

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composition via integrin receptors can modulate cell function (Schwartz et al. 1995; Woodard et al. 1998). However, regulation of MTJ structure in response to modified loading of fully differentiated muscle is not expected to be modulated by the application of ECM components to the MTJ membrane, because there is no evidence that the composition of the basement membrane at MTJs changes during modified muscle use. Thus mononucleated cell populations are more likely than the ECM components to exert any biological effects at MTJs.

The model employed in this study is an in vivo system in which muscles are unloaded by removal of weight-bearing and then subjected to renewed weight-bearing. Previous investigations have shown that this treatment results in remodelling of the MTJ, according to morphological criteria, and the presence of activated ED2+ macrophages at MTJs (St Pierre & Tidball, 1994a). It was tested whether (1) PDGF is synthesised by mononucleated cells at the MTJs of muscle experiencing reloading, (2) activated ED2+ macrophages can secrete PDGF at MTJs during modifications in muscle use, (3) PDGF receptors are internalised in reloaded muscle.

**Materials and Methods**

**Animal treatments**

Muscle unloading of adult female Wistar rats aged approximately 3 mo was achieved by hindlimb suspension using a modification (St Pierre & Tidball, 1994b) of the technique first developed by Morey-Holton & Wronska (1981). Six animals were suspended for a duration of 10 d followed by 2 d of reloading as the hindlimbs returned to weight bearing. Six ambulatory rats were used as controls. Both soleus muscles from ambulatory controls and animals reloaded for 2 d were dissected and 6 soleus muscles were used for immunohistochemistry or electron microscopy. All animal treatments followed protocols approved by the University of California, Los Angeles, Animal Research Committee.

**Immunohistochemistry**

Soleus muscles were dissected, frozen and sectioned at 10 µm as described previously (St Pierre & Tidball, 1994a). Longitudinal sections were then fixed with 2% paraformaldehyde in 50 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl (PBS) for 10 min before immunolabelling with rabbit anti-recombinant PDGF receptor (Upstate Biotechnology, NY, USA) diluted to 0.2 mg/ml in PBS and FITC-conjugated goat antirabbit IgG, according to previously described techniques (Tidball & Spencer, 1993).

Other longitudinal sections were fixed in methanol containing 0.3% H2O2 at −20 °C for 30 min and then treated with goat serum diluted for 30 min followed by a 30 min incubation in PBS. Those sections were immunolabelled for 2 h with rabbit anti-PDGF-BB (Genzyme, MA, USA) diluted 1:50 in PBS, rinsed in PBS for 30 min, incubated with PBS containing 0.1% bovine serum albumin, and then labelled with biotinylated second antibody according to previously described techniques (St Pierre & Tidball, 1994a). Longitudinal and cross sections of soleus muscles were also fixed with 2% paraformaldehyde in PBS, immunolabelled with an anti-ED2+ macrophages (Serotec, IN, USA) diluted 1:100 in PBS and FITC conjugated goat antimouse IgG (Sigma, MO, USA). Those sections were then immunolabelled with rabbit anti-PDGF (1:50) as described before. All sections were also incubated without primary antibodies to eliminate any artifacts.

The concentration of ED2+ and PDGF+ cells as well as internalised PDGF receptors was measured in 2 sections and randomly selected in each muscle used for immunohistochemistry and examined by light microscopy using Nomarski optics equipped with an eyepiece micrometer containing a 10 × 10 sampling grid. The number of labelled cells was counted manually by moving methodically the quadrant in each area to cover the whole section. The number of ED2+ and PDGF+ cells was counted in each section and the total area of the section determined and multiplied by its thickness in order to express the number of each cell type/mm3. The number of internalised PDGF receptors in each section was also counted and expressed as a percentage of total muscle fibres that terminated and contained a MTJ. The significance of differences between control and experimental samples was tested by the Mann-Whitney test, with the confidence limit set at P < 0.05.

**Electron microscopy**

Soleus muscles from ambulatory controls and animals reloaded for 2 d were then pinned near resting position and fixed in 1.4% glutaraldehyde in 0.2 M sodium cacodylate buffer at pH 7.2 for 1 h at 4 °C. Muscle samples were cut into ~ 5 mm3 pieces containing MTJs from the insertion end of the muscles, and fixed for an additional 30 min. The samples were rinsed in buffer and then postfixed in 1% osmium tetroxide for 40 min at 4 °C. Samples were dehydrated in a series of
ethanols and embedded in epoxy resin. Thick sections were evaluated by light microscopy so that longitudinally oriented samples could be selected for electron microscopy. Thin sections (70 nm) were cut longitudinally through muscle cells at MTJs, contrasted with uranyl acetate and lead citrate solutions. Regions containing MTJs were viewed at low magnification with a Zeiss EM 10A electron microscope. Those MTJs that were free from sectioning and processing artifacts were then photographed and analysed.

RESULTS

PDGF-expressing cells are present in reloaded muscle following hindlimb suspension

Indirect immunohistochemistry of frozen sections of soleus muscle using antibodies to PDGF-BB shows that control muscle contains infrequent PDGF+ cells in the tendon and in the connective tissue surrounding vasculature (Fig. 1). However, these PDGF+ cells in control muscles were not found to lie close to the surface of the muscle fibres or to be located near MTJs. PDGF+ cells were typically large mononucleated cells with irregular cell outlines, fusiform in shape, and resembling fibroblasts at MTJ sites. Soleus muscles collected from rats following 10 d of unloading by hindlimb suspension and 2 d of reloading contained significantly more PDGF+ cells than ambulatory control animals near the MTJs (Fig. 2). Tissue sections covering a surface area of ~36 mm² from reloaded muscles sampled near MTJs showed no ED2+ macrophages secreting PDGF. However, the ED2+ subpopulation of macrophages and PDGF+ cells were present at a density of 5510 ± 2258 cells/mm² and 70 ± 26 cells/mm², respectively. This density of PDGF+ cells is approximately 1% of the density of ED2+ macrophages at MTJs after 2 d of reloading following hindlimb suspension. No PDGF+ cells were observed to invade injured muscle fibres, which is a characteristic of the ED1+ subpopulation of macrophages that are present in rat muscles during reloading following hindlimb suspension (St Pierre & Tidball, 1994b).

PDGF receptors are internalised in skeletal muscle after 2 d of reloading

Muscles collected from healthy controls were observed to have PDGF receptors concentrated at MTJs of most muscle fibres (Fig. 3). The distribution of PDGF

Fig. 1. Light micrograph of section of control rat soleus muscle immunolabelled with anti-PDGF-BB and biotinylated second antibody, and viewed by Nomarski optics. A blood vessel lies in the centre of the micrograph (V), and is immediately surrounded by connective tissue, bordered by muscle fibres seen in longitudinal section. Arrows indicate some of the mononucleated cells containing PDGF-BB that lie in highest concentration near blood vessels and in the endomysium of control muscle. Bar, 50 μm.

Fig. 2. Light micrograph of longitudinal section of rat soleus muscle after 2 d of reloading following 10 d of unloading. The band of tissue on the left is tendon. The arrowheads outline the edge of a single muscle fibre that is terminating at an MTJ as it nears the tendon (T). The arrow indicates a mononucleated cell that is immunolabelled with anti-PDGF-BB and biotinylated second antibody. Bar, 50 μm.

Fig. 3. Longitudinal section of control rat soleus muscle immunolabelled with anti-PDGF receptor and fluorescent second antibody. (A) Section viewed by Nomarski optics showing tendon (T) to the left of the micrograph and the MTJ region of a single muscle fibre terminating at the tendon (arrowheads). (B) Indirect immunofluorescence image of same section showing PDGF-receptor at the MTJ. Bar, 50 μm.
Fig. 4. Longitudinal section of rat soleus muscle collected after 2 d of reloading following 10 d of unloading, and immunolabelled with anti-PDGF-BB and fluorescent second antibody. (A) Section viewed by Nomarski optics showing a nucleus (N), a perinuclear zone containing no myofibrils (between arrows) and several myofibrils. Four A-bands of an adjacent myofibril are indicated (arrowheads). (B) Indirect immunofluorescence from the same area shows several structures approximately 0.5 to 1 µm in diameter and containing PDGF-receptor lie in the perinuclear zone. Bar, 8 µm.

Fig. 5. Transmission electron micrograph of perinuclear region near an MTJ of a soleus muscle fibre following 2 d of reloading after hindlimb suspension. Note numerous lysosomes (arrows) that surround the nucleus (N). Bar, 1.5 µm.

receptors in these fibres resembled that of other proteins concentrated at MTJs that have been reported in previous studies (Tidball et al. 1986; Law et al. 1994). Muscles collected from rats following 2 d of reloading were also observed to have important concentrations of PDGF receptors at MTJs, although receptors were also observed highly concentrated in granules surrounding myonuclei near the MTJs of some muscle fibres (Fig. 4). These internalised receptors were observed after 2 d of reloading in approximately 5% of all fibres counted in the periphery of MTJs. Electron microscopic observations of MTJ regions after 2 d of reloading showed that nuclei near MTJs were associated with vesicles resembling lysosomes that were not found in control muscle samples (Fig. 5). The number of perinuclear vesicles observed by electron microscopy greatly exceeded the number of granules positive for PDGF receptor observed by indirect immunohistochemistry. This discrepancy suggests that only a minor fraction of the perinuclear vesicles contained internalised receptor, or the internalised receptor in most vesicles had been degraded so that the protein was no longer recognised by the antibody.

**DISCUSSION**

The findings of the present study show that mononucleated cells secreting PDGF are present at the MTJ during periods of modified loading. The presence of PDGF+ cells located at remodelling MTJs suggests that during modified loading, muscle fibres provide a signal that is either chemotactant or mitogenic for the PDGF synthesising cells. ED2+ macrophages, which are a subpopulation of macrophages that reside normally in muscle and other tissues (Honda et al. 1990; McLennan, 1993, 1996), have been shown to increase in concentration and become activated, as assessed by morphological criteria, during skeletal muscle reloading following hindlimb suspension (St Pierre & Tidball, 1994a). A recent study has shown that ED2+ macrophages can release various growth factors that promote myoblast proliferation and myotube formation in vitro (Massimino et al. 1997). Macrophage populations are capable of secreting the PDGF molecule (Shimokado et al. 1985) which can elicit different biological responses on skeletal muscle cells including increased chemotaxis (Venkatasubramanian & Solursh, 1984) and mitogenic activities (Yablonka-Reuveni et al. 1990; Yablonka-Reuveni & Seifert, 1993; Yun et al. 1997) as well as inducing cytoskeletal reorganisation (Tidball & Spencer, 1993). However, ED2+ macrophages do not produce the
isoform PDGF-BB in reloaded muscle so that other mononucleated cells synthesise PDGF-BB near MTJs during changes in muscle use. The finding that cells of fusiform shape or irregular outline that resemble fibroblasts secrete PDGF at MTJs after 2 d of reloading is consistent with the electron microscopic observations that mononucleated cells with extensive rough endoplasmic reticulum are present at MTJs injured by overloading (Zamora & Marini, 1988). Fibroblasts are likely to be important in regulating muscle regeneration and the remodelling of MTJs, in that they have been shown to secrete a wide range of cytokines of major importance during inflammatory responses (Smith et al. 1997). The PDGF+ cells at the MTJ are viewed as the feasible source of PDGF applied to the MTJ because no other local sources were identified in the present investigation. Also, PDGF has a brief half-life in vivo (Bowen-Pope et al. 1984), so that release from a remote site followed by diffusion to the MTJ is unlikely. Unfortunately, fibroblast populations consist of subsets of cells displaying different phenotypes and as there are no specific markers for fibroblasts in vivo, their identity cannot be conclusively established.

Our immunohistochemical observations show that only ~5% of muscle fibres in a section of reloaded muscle contain internalised PDGF receptors. Internalisation of PDGF receptors into lysosomes has only been shown in previous studies to occur following PDGF binding (Heldin et al. 1981; Nilsson et al. 1983; Rosenfeld et al. 1984). The internalisation of PDGF receptors at nuclei near the MTJs supports the hypothesis that PDGF is released from mononucleated cells at the reloaded MTJ, and is then bound by PDGF receptors at the MTJ and internalised. The binding of PDGF to its receptor can have a pleiotropic action on the target cells. Indeed, PDGF stimulation of cells in vitro can cause the release of calcium from intracellular stores (Moolenaar et al. 1984), the influx of extracellular calcium into the cell (Diliberto et al. 1992), phosphatidyl inositol turnover (Habenicht et al. 1981), activation of protein kinase C (Kazlauskas & Cooper, 1988) and release of protein kinase A from the cell membrane (deBlaquiere et al. 1994). All these responses have been associated with cytoskeletal rearrangements in some cells under at least some conditions. For example, evidence acquired in vitro supports the view that PDGF molecules are capable of regulating cytoskeletal-membrane interactions at the focal adhesion site, an analogous structure to the MTJ in vitro (Tidball & Spencer, 1993). Furthermore, PDGF may influence MTJ structure by less direct mechanisms that involve modification in gene expression (Lee et al. 1987). PDGF has been shown to stimulate the phosphorylation of transcriptional factors and to induce or modulate the expression of several genes, which may have unknown and indirect effects on MTJ structure (Muller et al. 1984; Bu & Hagedorn, 1991).

In conclusion, this study provides evidence that PDGF+ cells are closely apposed to the muscle cell surface near MTJs and that irregular fusiform cells resembling fibroblasts are the most likely candidates for the production of PDGF molecules. The PDGF+ cells may be involved in regenerating and remodelling processes occurring after muscle injury. Furthermore, the presence of a high concentration of PDGF+ cell at MTJs after 2 d of reloading coincides with internalisation of PDGF receptors which suggest their activation and possible biological actions at MTJs in response to changes in the mechanical environment.

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