Critical Limb Ischaemia in Adult Human Skeletal Muscle Increases Satellite Cell Proliferation but Not Differentiation

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Abstract

Critical Limb Ischaemia (CLI) is defined as the presence of rest pain, ulcers and/or gangrene in a limb for over 2 weeks. Associated exercise intolerance is caused by muscle fibre atrophy, fibro-fatty infiltration, nerve dysfunction, mitochondrial damage and myofibril disorder. We aimed to determine the behaviour of satellite cells, responsible for the repair and regeneration of damaged muscle, in repairing the damage caused to critically ischaemic adult human skeletal muscle. CD34, pax7 and MyoD are all markers of satellite cells at different stages of the cell cycle and allow for an assessment of their number and activity in ischaemia. Local ethical committee approval and informed consent was obtained. Samples of harvested gastrocnemius muscle of patients undergoing major perigenicular amputation for CLI (n = 10) were analysed and compared to a control group undergoing coronary artery bypass grafting (n = 10). Using immunohistochemistry, the expression of pax7, CD34 and MyoD was assessed in five sequential blinded randomly generated fields. Statistical testing of the data collected was made via the Mann Whitney U test. Protein electrophoresis was used to confirm overall protein expression of the satellite cell markers. There was a significant increase in the number of satellite cells observed in CLI muscle sections as demonstrated by the expression of pax7 (2.4× fold p < 0.0001). CD34 expressing Haematopoietic Stem Cells (HSCs) and satellite cells were also more abundant, with a 2× fold increase observed (p < 0.0001) whilst those cells expressing both CD34 and pax7 and identified as quiescent satellite cells, were significantly greater in number in the CLI samples (2.9× fold p < 0.0001), confirmed via immunohistochemistry and protein electrophoresis. There was a significant decrease in the expression of MyoD positive or activated satellite cells (p < 0.0001). This indicates an increase in the proliferation of the satellite cell population as a response to CLI but less active cells are observed.

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Keywords
Muscle, Stem Cells, Satellite Cells, Muscle Progenitor Cells, Ischemia, Gastrocnemius

1. Background

Critical Limb Ischæmia (CLI) is defined as the presence of symptomatic limb ischæmia of over 2 weeks duration usually caused by the presence of increasing intraluminal atheroma. It affects between 500 - 1000 patients per million annually [1]. Atherosclerotic lesions reduce blood flow to large muscle end organs such as the thigh, calf and buttocks resulting in intermittent claudication on exercise. Prolonged reduction in blood flow, insufficient to deliver oxygen and nutrients also leads to lactic acidosis which in turn causes pain. Worsening atherosclerosis over a prolonged period causes rest pain in the extremities, relieved by gravity dependence and leading to patients sleeping overnight in chairs [2]. As plaques increasingly occlude the lumen of blood vessels over protracted periods of time, leading to tissue ulceration and ultimately gangrene [3].

Reduced exercise tolerance is caused by muscle fibre atrophy, fibro-fatty infiltration, nerve dysfunction, mitochondrial damage and myofibril disorder [4] [5]. An abundance of hybrid fibres, containing two myosin heavy chain phenotypes, has been observed in ischæmic tissue and indicates a dynamic process of fibre type repair, shifting and myofibril regeneration in ischæmic muscle [6] [7]. Type I fibres more resistant to hypoxia become predominant as type IIx fibres are preferentially lost [4]-[6] [8]-[11]. The behaviour of satellite cells in critically ischæmic human skeletal muscle has not been well characterised, but these cells are responsible for the normal processes of repair and regeneration of damaged muscle including that caused by ischæmia [12]-[14]. Due to the deterioration in exercise tolerance seen in CLI, the morphological changes that take place indicate that satellite cells become dysfunctional or their regenerative capacity becomes overwhelmed.

Treatment of peripheral vascular disease requires a multi-disciplinary approach involving the treatment of atherosclerosis itself, through the eradication of its risk factors and symptom control [1]. Therapeutic agents have had limited success in treating CLI and endovascular and open surgical procedures have poor outcomes [1] [2]. In treating ischæmic and perhaps other myopathies, it is possible that a therapeutic answer will lie with the administration of pro-myogenic agents administered to promote satellite cell function, grown in vitro and implanted into diseased muscle [14]-[16]. It has been shown that the precursors of these satellite cells, either derived from resident “side population cells” (SPCs) [17] [18] or circulating bone marrow derived haematopoietic stem cells (HSCs), are responsible for the myogenic process and may even have the potential to contribute to the processes of neurogenesis and angiogenesis [15] [16] [19]-[21]. Increased angiogenesis is certainly observed in ischæmic tissue, mediated through the global actions of Hypoxia-Inducible Factor (HIF)-1α. However the newly formed vessels do not reach maturity and are not fit for purpose, lacking a stable structure seen in established mature vessels [22]. Although satellite cells are by definition a myogenic precursor cell with their fate predetermined by the expression of pax7, they have been shown to be capable of regenerating cardiomyocytes in vitro and they may have the potential to form other mesodermal tissue [15] [16] [23].

Table 1 shows some commonly identified protein markers used to identify satellite cells at different stages in their life cycle. There is no single marker available to label all satellite cells, throughout the different stages of their cell cycle [24] but the majority of active, quiescent and proliferating satellite cells express pax7 [25]-[27] a paired-box transcription factor that has a molecular weight of 57 kDa and is essential for the mitogenesis of these cells and the formation of either myogenic cells or rejuvenating the satellite cell pool [25] [28]-[30]. It is co-expressed with MyoD or myf-5 in activated satellite cells but is not present in those cells expressing myogenin [30] [31]. Quiescent satellite cells are pax7+/CD34+/MyoD−, whereas most active and proliferating cells express pax7+/CD34+/MyoD+ [24]-[30] [32]-[34].

This study aimed to show how by using some of the markers specified above, in an otherwise well matched sample of muscle biopsies, the presence of CLI affects satellite cell number and activity in vivo.

2. Materials and Methods

2.1. Study Population

Ten patients undergoing major lower limb amputation for critical limb ischæmia and ten age-matched patients...
undergoing coronary bypass grafting were recruited to this study after local ethical committee approval and informed consent was obtained. Patients with acute or acute on chronic ischaemia, auto-immune disease including vasculitides or tissue necrosis or oedema close to the site of biopsy were excluded from this study. Table 2 displays the demographics, risk factors for vascular disease and co-morbidities of the patients involved in this study.

2.2. Tissue Collection and Processing

The medial head of gastrocnemius \[35\] was biopsied in all cases and preserved immediately after harvesting by immersion either in liquid nitrogen or in formalin and later wax embedded. Paraffin embedded tissue was cut into 3 μm slices perpendicular to the fibres using a microtome and eight consecutive slides were prepared in this way for picrosirius staining to assess orientation and identification of CD34, pax7 and myoD antibodies through standard ABC immunohistochemistry.

Five randomly chosen but sequential 400 × 300 μms fields, following established protocols \([5]-[15]\) \([17]-[36]\) were identified on pre blinded slides and analysed using the Zeiss Axiosopp 4 light microscope at 200× magnification. Image capture was performed using an Olympus camera and JPEGs generated were stored to be analysed. The ABC method of immunohistochemistry was utilised with DAB chromagen and positive stains for each antibody were recorded in sequential slides and subjected to data analysis as outlined below. Table 3 illustrates the

### Table 1. Satellite cell markers \([14]\).

Some of the many markers expressed by satellite cells during different stages in their life cycle, with an indication of when these are expressed (+) or absent (−). In this present study, three protein markers (one cell surface protein and two transcription factors) were used for ease of analysis and because they complement each other in identifying the relevant cells as detailed later.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Quiescent</th>
<th>Active</th>
<th>Proliferating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Surface</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>c-met</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M-cadherin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MNF</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Transcription Factor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>myf5</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MyoD</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pax7</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

### Table 2. Patient demographics: There was no significant difference in the mean age of the two cohorts. (Mann Whitney U test; \(p < 0.05\) inferred significance; 95% CI). Patients were otherwise well matched for co-morbidities, risk factors as well as demographic characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Ischaemics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>73.20 ± 1.965 N = 10</td>
<td>73.60 ± 2.405 N = 10</td>
</tr>
<tr>
<td>Sex</td>
<td>9M:1F</td>
<td>8M:2F</td>
</tr>
<tr>
<td>Dyscrasias</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Diabetes</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Heart Disease</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>CVA/ TIA</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Hypertension</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>PAD</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Renal Disease</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>AAA</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Smoking history</td>
<td>8</td>
<td>9</td>
</tr>
</tbody>
</table>
Table 3. Panel of antibodies used for satellite cell recognition.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Source</th>
<th>Species</th>
<th>Conc.</th>
<th>Pre-Treatment</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>QBEnd-10</td>
<td>Dako</td>
<td>Mouse monoclonal IgG</td>
<td>1:50</td>
<td>EDTA 20m</td>
<td>RT 1h</td>
</tr>
<tr>
<td>Pax7</td>
<td>QC2152</td>
<td>Aviva Systems</td>
<td>Rabbit Polyclonal</td>
<td>1:200</td>
<td>No PT</td>
<td>4°C ON</td>
</tr>
<tr>
<td>MyoD</td>
<td>5.8A</td>
<td>Dako</td>
<td>Mouse monoclonal IgG</td>
<td>1:50</td>
<td>EDTA 20m</td>
<td>4°C ON</td>
</tr>
</tbody>
</table>

anti-bodies used and their optimised protocols, subject to negative controls and used as outlined below with development of DAB chromagen (Vector Laboratories Ltd.) for analysis with haematoxylin counter staining.

2.3. Protein Analysis

Protein expression of CD34, pax7 and MyoD in both sample groups was quantified using the Western blot technique with the aforementioned specific primary antibodies probing for their particular antigens and detection via densitometry. 50 µls Homogenising buffer (25 mM Tris/HCl pH = 7.4, 1 mM EDTA and 1 mM EGTA) was added to each sample. The cell extracts were then centrifuged to pellet cell debris. Supernatants were added to 20 µls of 4× loading buffer (20% sodium dodecyl Sulphate, 1 M Tris/HCl pH 6.8, glycerol, mercaptoethanol, 0.2% bromophenol blue and deionised water) and heated in a water bath at 90°C for 5 minutes. Total protein (20 µgs) and 10 µls of a molecular weight marking ladder were separated by SDS-PAGE electrophoresis with a current of 125 mV over 90 minutes applied to 4% - 20% Tris glycine polyacrylamide gels and transferred to nitrocellulose membrane (Hybond C-Extra membrane—Amersham Biosciences UK) by electroblotting using a transfer buffer (50 mls methanol, 20 mls 25× Tris Glycine Buffer, made up to 500 mls with deionised water) and a current of 35 mV for 90 minutes. The membranes were blocked in 5% milk in PBS/Tween for an hour followed by incubation with specific primary antibodies overnight at 4°C. The following primary antibodies were diluted in 1× TRIS buffered saline to be used for immunoblotting at the following concentrations: pax7 (Aviva Biosystems) 1:2000, CD34 (Dako [1:2000] and MyoD (Dako) 1:1000.

The appropriate secondary horseradish peroxidase (HRP)-conjugated affinity purified goat anti-rabbit for pax7 and HRP-conjugated goat anti mouse for CD34/MyoD (Vector laboratories) was added to the membrane at the consecutive concentrations of 1:3000 and 1:2000 at room temperature for an hour. Over half an hour a TBS wash was applied every ten minutes and developed after the addition of Enhanced Chemiluminescence Kit, (Amersham Biosciences UK) to the membranes placed on autoradiographical film (ECL Hyperfilm, Amersham Biosciences UK). Quantification of the probed antigens was performed via a laser scanner and these bands were expressed in relative density units (RDU) according to the strength of the luminescence and represented in Figure 2.

3. Results

CD34 is well known to be expressed by both satellite cells and haematopoietic stem cells (HSCs). Pax7 is expressed by satellite cells alone. The combination of the two, co-localised, highlights quiescent satellite cells in sequential slides. MyoD is a marker of activated satellite cells [25]-[34].

Expression of pax7 was found to be markedly increased throughout the ischaemic muscle samples when compared to the control group samples. Not only was there a 2.4 fold increase in pax7 positively staining nuclei of satellite cells per field (p < 0.0001), but it was noted that there was a clear difference in the pattern of expression seen by greater amounts of the brown DAB chromagen throughout the myofibrils. CD34 positive nuclei in ischaemic tissue were found to be increased by twofold, (p < 0.0001) when compared to control tissue. CD34 and pax7 positive cells were noted to be increased in ischaemic tissue when compared with control tissue (2.9× fold increase p > 0.0001; 95% CI). Figure 1: Serial sections from an ischaemic sample vs a control sample for pax7, CD34 and MyoD. Graphs c, f and i also show the mean number of positive nuclei/per five fields of each sample.

The over expression of pax7 and the increased CD34 seen in immunohistochemistry was then quantified via Western Blotting, the results are displayed in Figure 2. Conversely, while expression of MyoD in control tissue is scant, there is a significant decrease ×1.75 (p < 0.0001) in ischaemic tissue compared to control.
Figure 1. (a)-(i) pax7 expression in Control Muscle (b) pax7 expression in Ischaemic Muscle (200× magnification using DAB chromagen). There is over expression of pax7 as well as a 2.4× fold mean increase in positive staining cells throughout the ischaemic tissue (p < 0.0001) represented in graph 1(c); Sequential Slides showing CD34 expression in (d); Control Vs (e) Ischaemic Tissue. There was 2× fold increase in mean CD34 positive cells (p < 0.0001) in the latter represented in (f); MyoD expression in control tissue (g) vs ischaemic tissue (h) and significantly reduced 1.75× (p < 0.0001) MyoD expression in ischaemic tissue, is represented in Graph (i).

Over expression of pax7 seems to have a negative effect on MyoD expression, seen in other studies [30] [37] [38]. Western blots of CD34, pax7 and MyoD were performed in frozen samples of both groups and expression of the proteins is represented below.

4. Co-Localisation of CD34 and Pax7 Using Sequential Slides

To confirm the activity of the numerous satellite cells observed, five random fields per sample on sequential slides were analysed and the number of cells staining for both CD34 and pax7 but not MyoD, which when co-localised, identified quiescent satellite cells. There was a mean of 6.3 cells staining positive for both CD34 and pax7 per 400 × 300 μms field in control tissue compared with 18.46 cells per field in ischaemic tissue, a 2.9× fold increase (p < 0.0001). Figure 3 represents the abundance of quiescent cells in ischaemic vs control tissue with representative stains of a single field with CD34/pax7 but an absence of MyoD.
5. Conclusions

Satellite cells resident within skeletal muscle can be truly described as stem cells having a multipotent fate. It is these therefore that represent a source of autologous repair for damage caused by ischaemia. Pax7 is crucial to bestow a myogenic fate on satellite cells, myogenic precursors and MyoD is further required to commit myoblasts to a myogenic lineage [14] [39] [40]. In this study, an abundance of pax7 is seen throughout ischaemic tissue. Satellite cells expressing pax7 and CD34 and not myoD were present in ischaemic muscle in greater numbers. These represent the population of quiescent satellite cells or reserve cells, which are myogenic precursor cells and have the ability to form either myofibrils or satellite cells [17]. Their numbers as identified by pax7, MyoD and CD34 in this study are greatly increased in ischaemia but exist in an inactive state. It is likely that ischaemia induces proliferation and inhibits differentiation as evidenced by the reduction in cells expressing MyoD.

Activated satellite cells marked by pax7 and MyoD alone, are present but scarce in critical ischaemia. They may be the only cells which are responsible for renewing the satellite cell pool [30]. Derived from local skeletal muscle side population cells or from circulating haematopoietic cells, they become committed to a myogenic fate due to the high levels of pax7 present rather than to replenish the satellite cell pool. Other groups have found, in the damaged or denervated muscle of rodents at least, that MyoD and myogenin accumulation is prevalent [40]. Interestingly, this was not observed in this present study. CLI induced damage in adult human skeletal muscle significantly decreased in protein expression in MyoD.

6. Discussion

The muscle regulatory factors (MRFs), including MyoD, are able to dictate a skeletal muscle phenotype to non-specific progenitor cells. Proliferating myoblasts express myoD and myf5 in their cytoplasm before the
stage of differentiation and those satellite cells differentiating into myofibres or satellite cells nuclei express MyoD (37). Interestingly, MyoD was less abundant in CLI tissue. The up regulation of the MRFs then leads to the exit of either the myoblasts or satellite cells from the cell cycle; myogenin is expressed and perpetuates the process of differentiation [33] [41]. Pax7 over expression seen here in CLI is similar to that reported by Enns and Tidus [37] and others [25] [30] which caused proliferation and inactivation of satellite cells but not their differentiation due to inhibition of MyoD [38]. Such activity prevents exit from the cell cycle which is required for cellular differentiation [41]. The process of myogenesis in adult ischaemic tissue may be incomplete as while satellite cells increase in number, these are generally not activated cells and cannot achieve maturity or differentiation [42].

Further work to elucidate the pathway that activates and causes the proliferation of quiescent satellite cells into myoblasts or renews the population of these active myogenic precursors would be the next logical step. The processes required stimulating the mesodermal progenitor cells to produce satellite cells, or those required to activate the numerous resident myogenic precursor cells will be crucial in combating the poor response of myogenic repair and regeneration seen in critical limb ischaemia. They may also serve as a source of stem cells which can contribute to new vessel, muscle and even nerve growth [16] [39] [40] [43]-[45].

References


