Transgenic expression of human complement regulators reduces skeletal muscle ischaemia/reperfusion injury in mice

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ABSTRACT

This study aimed to explore the hypothesis that activated complement components contribute significantly to I/R (ischaemia/reperfusion) injury in skeletal muscle. After 50, 70 and 90 min of tourniquet ischaemia and 24 h of reperfusion, viability of the medial gastrocnemius muscle in C57BL/6 wild-type mice, assessed histochemically by reduction of NBT (Nitro Blue Tetrazolium) dye, was 60, 21 and 8% respectively. Skeletal muscle viability after 70 min of ischaemia and 24 h of reperfusion in transgenic mice expressing a combination of human CD46, CD55 and CD59, all inhibitors of complement activation, was 45% compared with 24% in ischaemic reperfused wild-type mice (P = 0.008; n = 6 per group). Muscle from sham-treated transgenic mice and wild-type littermates had no significant loss of viability relative to normal contralateral gastrocnemius muscle. A significant reduction in myeloperoxidase activity (a measure of neutrophil infiltration), xanthine oxidase activity (a source of free radicals) and water content (a measure of oedema) was observed in ischaemic reperfused muscle from transgenic mice compared with ischaemic reperfused wild-type muscle (P < 0.05). Haematoxylin and eosin-stained histological sections also showed less damage and less apparent leucocyte infiltration in muscles from ischaemic reperfused transgenic mice than those from wild-type animals given the same degree of injury. Muscles from sham-treated transgenic and wild-type controls were almost identical with normal muscle. It is concluded that complement activation contributes to the pathogenesis of I/R injury in murine skeletal muscle, resulting in increased neutrophil infiltration into the injured muscle, increased free radical production and vascular permeability during reperfusion, and a net detrimental effect on muscle viability.

INTRODUCTION

Necrosis of skeletal muscle after I/R (ischaemia/reperfusion) injury, as in replantation and crush injuries to limbs and skin flaps, is a major problem in plastic and reconstructive surgery. During ischaemia there is cell swelling, hypoxia, lactic acidosis and depletion of tissue levels of ATP. During reperfusion, vasoconstriction, thrombosis, oedema, increased infiltrating leucocytes and increased concentration of free radicals all contribute to eventual tissue necrosis [1,2].

Complement activation appears to play an early role in reperfusion injury, both directly and indirectly, via its effects on neutrophils, endothelial cells and mast cells [3].
In skeletal muscle, activation of complement leads to formation of anaphylatoxins such as C3a and C5a, which cause increased microvascular permeability [4], neutrophil sequestration [5], up-regulation of neutrophil receptors such as CD11b/CD18 [6] and stimulation of neutrophils and other leucocytes to elicit the release of oxygen free radicals, proteolytic enzymes, pro-inflammatory cytokines, such as IL-1 (interleukin-1) and TNF-α (tumour necrosis factor-α), and other inflammatory mediators, including NO (nitric oxide)-generated peroxynitrite and PAF (platelet-activating factor) [7].

The complement system is a cytotoxic host defence mechanism which, when activated, results in increased vascular permeability, chemotaxis and opsonization. It comprises 20 proteins, all of which are constitutively expressed in the blood and contribute to a cascade of reactions that culminate in the formation of a terminal MAC (membrane attack complex) [8,9]. MAC is a large molecular complex including the proteins C5b, C6, C7, C8 and C9. Its function is to form transmembrane channels in cells, leading to lysis and cell death [9]. It is normally activated to provide defence against micro-organisms and antigen–antibody complexes, although inappropriate activation during I/R can lead to further damage of ischaemic tissue [10,11].

There are two major pathways through which complement acts: the classical pathway (activated by antigen–antibody complexes) and the alternative pathway (activated by micro-organisms). Both pathways terminate with MAC. There is no definitive answer as to which pathway is involved in I/R injury, as many conflicting results have been published to date [12–17]. However, the strongest evidence for I/R injury in skeletal muscle supports the hypothesis that complement acts through the classical pathway [14,18].

It is not known whether complement initiates I/R injury or is activated by inflammatory mediators during reperfusion of ischaemic tissue. It is thought that activation of complement could be initiated in response to subcellular membrane fragments and organelles released from damaged endothelial cells during ischaemia [12,13]. There is recent evidence, however, that IgM binding to ischaemic skeletal muscle precedes the damaging complement activation by a significant period of time [19].

The present study is based on the hypothesis that activated complement components contribute significantly to I/R injury in skeletal muscle. The major aim was to determine whether expression of the human complement activation inhibitors CD46 (membrane cofactor protein), CD55 (decay accelerating factor) and CD59 (protectin) in transgenic mice affects the viability of murine skeletal muscle following I/R injury. There is evidence that all three human molecules are capable of regulating rodent complement (Figure 1) [20–23]. Compared with experiments in the past using non-specific inhibitors of complement activation, the use of transgenic mice expressing these molecules has provided more definitive answers to the role of complement activation in I/R injury.

**METHODS**

**Materials**

Transgenic mice exhibiting widespread tissue expression of human CD46, CD55 and CD59, driven by the mouse H2Kb promoter, were generated on a CBA-C57BL/6 background. The form of CD46 expressed was associated with the cell membrane by a glycosyl-phosphatidyl inositol linkage, rather than the native transmembrane domain. This modification does not interfere with the complement regulatory function of CD46 [20]. The major chemicals were purchased, except where indicated, from Sigma (St Louis, MO, U.S.A.) or from Aldrich (Castle Hill, New South Wales, Australia).

**I/R model in the mouse hind limb**

Pilot study: the ideal ischaemia time for the mouse model

In a preliminary study, CBA-C57BL/6 mice, weighing 20–28 g and acclimatized to a 12 h day/night cycle for 7 days prior to use, were anaesthetised with 4% chloral hydrate in normal saline at 0.1 ml/10 g of body weight. Mice were given 50, 70 or 90 min of ischaemia followed by 24 h of reperfusion (n = 6 per group). Ischaemia was induced by application of an elastic rubber band (size 8, four turns) as high as possible on the right thigh, as described previously [24]. A thin needle thermistor probe was inserted through the skin on the right thigh directly into the gastrocnemius muscle. The mouse was placed in a box heated by a lamp with the head shielded from direct light. By manually adjusting the distance of the lamp from the mouse, it was possible to maintain the muscle temperature at 36 ± 1°C. After removal of the tourniquet, the mice were allowed to recover from anaesthesia and reperfusion continued at normal body temperature for 24 h.
The mice were re-anaesthetized and the medial gastrocnemius muscles from both the ischaemic and contralateral legs were harvested and immediately cooled to 4 °C.

The main study
The following mice were anaesthetized and subjected to 70 min tourniquet ischaemia at 36 °C and 24 h reperfusion at ambient temperature (21 °C), as described above: (i) CD46/CD55/CD59 transgenic mice (H2Kβ promoter) bred on a CBA-C57BL/6 background (n = 6); and (ii) wild-type CBA-C57BL/6 littermates (n = 6).

For sham-operated control groups, mice were anaesthetized with 4% chloral hydrate, left at 36 °C for 70 min without a tourniquet, then allowed to recover for 24 h at room temperature: (iii) CD46/CD55/CD59 transgenic mice (H2Kβ promoter) bred on the same CBA-C57BL/6 background as the wild-type mice (n = 6); and (iv) wild-type CBA-C57BL/6 littermates (n = 6).

At the end of the treatment period, all mice were re-anaesthetized and gastrocnemius muscles harvested from both hind limbs. Each muscle was divided into five equal slices of approx. 2 mm in width. Slices 2 and 4 were taken for muscle viability determination by the NBT (Nitro Blue Tetrazolium) method and histological analysis; slices 1, 3 and 5 were freeze-dried and stored at −70 °C for wet weight/dry weight ratios and biochemical analysis.

Muscle viability
NBT, a histochemical dye which is oxidized from colourless to a dark blue colour by viable mitochondrial enzymes, was used to identify the proportion of each muscle slice that was still viable after I/R injury. This assay, first developed using canine gracilis muscle by Labbe et al. [25], has been adapted in our laboratory to determine viability in rabbit rectus femoris [26], as well as rat [27] and mouse [24] gastrocnemius muscle. Hickey et al. [26] determined that the optimal time to assess muscle viability was 24 h post-ischaemia, as this correlated well with the ultimate regeneration of the I/R-injured muscle 1–2 weeks later. We therefore adopted 24 h reperfusion as the standard time for this assay in these experiments. The optimized conditions as they apply to the mouse model have been described in detail elsewhere [24,27]. The critical difference was the incubation time of muscle in NBT solution of 10 min at 21 °C for mice compared with 20 min incubation time when using thicker slices of muscle from rabbits and rats.

Freshly harvested muscle slices were incubated in 3–5 ml of a solution of NBT dye (0.033%, w/v) and NADH (0.133%, w/v) in 0.05 M sodium phosphate buffer (pH 7.4) for 10 min at ambient temperature (21 °C) and fixed in 4% (v/v) paraformaldehyde in 0.01 M PBS (pH 7.4). To determine the percentage of viable tissue from each muscle, a non-magnified image of each muscle slice was transmitted to an IBM computer by a CCD video camera (WV-CL700A; Panasonic, Osaka, Japan). The image was analysed with the software package Video Pro 32 (Leading Edge, Marion, South Australia, Australia). Both faces of each muscle were analysed for viability (percentage area of blue-stained muscle). The percentage of viable muscle was determined by averaging the percentage area of each individual muscle face. These semi-automated analyses were carried out by an operator blinded to the treatment of the specimens. The results from all muscle faces in each group were averaged and expressed as a percentage of the viability of the contralateral control.

Biochemical analysis of muscle homogenates
Wet muscle tissue was weighed, freeze-dried for 2 days and then reweighed. Tissue water was calculated as the mass of water (wet weight minus dry weight) per g of dry tissue [27].

The degree of neutrophil infiltration in the muscle samples was estimated by measurement of muscle MPO (myeloperoxidase) activity [27,28]. Freeze-dried muscle in liquid nitrogen was mashed into a powder with a mortar and pestle. A portion (10–20 mg) of dry tissue was weighed, homogenized (Ultraturrax TT25 homogeniser; IKA Laboratechnik, Brussels, Germany) for 30 s in 0.02 M potassium phosphate buffer and centrifuged at −20 °C for 2 min at 2135 g (Sorvall RT 6000D; DuPont, Melbourne, Australia). The supernatant was discarded and the pellet fraction re-homogenized in 0.05 M potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. The samples were freeze/thawed twice and re-centrifuged as above but at 12 °C. An aliquot of supernatant was assayed by measuring the H2O2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine in 50 mM sodium acetate buffer (pH 5.4) containing 0.6 M sucrose. One unit of enzyme activity was defined as the change in absorbance/min at 655 nm at 37 °C per g of dry muscle tissue. The intra-assay CV (coefficient of variation) at 8 units/g was 11.3%.

XO (xanthine oxidase) activity was determined using an established method [29]. Briefly, 10–20 mg of tissue was homogenized in 50 µl of 0.05 M Tris/HCl buffer (pH 7.2), followed by the addition of reagent mixture (950 µl) containing 20 µM 2-amino-4-hydroxypyridine, 1 mM EDTA, 10 mM dithioerythritol and 1 mM PMSF (all obtained from Sigma). The mixture was incubated in a shaking water bath at 37 °C for 30 min. Standard and blank tubes were placed in boiling water for 3 min to stop the enzyme reaction and centrifuged for 10 min at 1500 g. A portion (500 µl) of supernatant was diluted with 0.1 M sodium acetate buffer (pH 5.4) and placed in a fluorimeter set at 347 nm excitation and 405 nm emission. Activity was calculated as pmol of isoxanthopterin.

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Table 1  Tissue water content and MPO and XO activities in skeletal muscle homogenates following either sham operation or I/R (70 min of ischaemia/24 h of reperfusion)

Table:  

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<th>Sham-operated muscle</th>
<th>Ischaemic-reperfused muscle</th>
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<td></td>
<td>CD46/CD55/CD59 transgenics</td>
<td>Wild-type littermates</td>
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<td></td>
<td>CD46/CD55/CD59 transgenics</td>
<td>Wild-type littermates</td>
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<tr>
<td>Water content (ml/g)</td>
<td>3.67 ± 0.17</td>
<td>4.08 ± 0.23</td>
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<tr>
<td>MPO activity (units/g)</td>
<td>1.11 ± 0.23</td>
<td>1.31 ± 0.17</td>
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<tr>
<td>XO activity (pmol · h⁻¹ · g⁻¹)</td>
<td>9.5 ± 1.4</td>
<td>8.1 ± 0.9</td>
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Values are means ± S.E.M. of six experiments. ∗P = 0.015, †P = 0.032 and ‡P = 0.012 compared with I/R-treated wild-type littermates.

formed · h⁻¹ · g⁻¹ of wet weight. The intra-assay CV at 18 pmol · h⁻¹ · g⁻¹ of wet weight was 8.9 %.

Histology and morphology
The specimens of each muscle used previously in the NBT assay (slices 2 and 4 and, in some mice, slice 3) were fixed with 4 % (v/v) paraformaldehyde in 0.01 M PBS (pH 7.4) for 3 h, embedded in paraffin, and 5 µm sections were cut and stained with haematoxylin and eosin.

Statistical analyses
Results are expressed as means, together with the S.E.M., and the numbers of animals per group. Data were compared by an ANOVA, and the significance of the differences between groups was assessed by Dunnett’s correction for multiple comparisons (GraphPad Prism software, San Diego, CA, U.S.A.).

RESULTS

Muscle viability following variable ischaemia (50–90 min) and 24 h of reperfusion
In a pilot study designed to determine a model with severe, but potentially recoverable, muscle injury, we measured muscle viability by the NBT method following increasing durations of ischaemia with a fixed duration of reperfusion. A period of 50 min of ischaemia/24 h of reperfusion resulted in 60 % muscle viability and 70 min of ischaemia/24 h of reperfusion resulted in 21 % muscle viability, whereas 90 min of ischaemia/24 h of reperfusion resulted in 8 % muscle viability. We concluded that 70 min of ischaemia/24 h of reperfusion best fulfilled these criteria, and these conditions were adopted in subsequent experiments.

Effect of inhibition of complement activation using CD46/CD55/CD59 transgenic mice
In the main study, viability of gastrocnemius muscle in wild-type mice after I/R (70 min of ischaemia/24 h reperfusion) was 24 ± 4 %. This improved significantly (P = 0.008) to 45 ± 4 % in muscle from CD46/CD55/CD59 transgenic mice subjected to the same I/R injury. Sham treatment did not cause any noticeable loss of muscle activity in either wild-type or transgenic muscle, with these muscles having 98 ± 1 % and 96 ± 1 % viability respectively, relative to normal contralateral gastrocnemius muscle.

Tissue MPO and XO activity and water content
In I/R-injured skeletal muscle from CD46/CD55/CD59 transgenic mice compared with those in I/R-injured muscle from wild-type mice, inhibition of complement activation led to small, but significant, reductions in MPO (P = 0.032) and XO (P = 0.012) activities (Table 1). There was also a significant reduction (P = 0.015) in water content in the I/R-injured muscle in CD46/CD55/CD59 transgenic mice compared with wild-type mice (Table 1). In the sham-operated mice, levels of all three parameters were not significantly different in transgenic muscle compared with wild-type muscle (Table 1).

Histology of I/R-injured muscle
Sham-operated muscle in wild-type and transgenic mice was similar in appearance to normal muscle, with strong staining of the muscle fibres and cytoplasm and nuclei located in the periphery of these cells (Figures 2a and 2b). After 70 min of ischaemia/24 h of reperfusion, the muscle fibres and cytoplasm of specimens from wild-type mice were more weakly stained, the peripheral nuclei were less distinct or absent, and the interstitial spaces were filled with many leucocytes, mostly neutrophils (Figure 2c). After the same degree of I/R, muscle from the transgenic mice was similar in appearance to the controls, except for the presence of neutrophils and other leucocytes (Figure 2d). Although there appeared to be slightly fewer leucocytes in transgenic muscles compared with wild-type muscles, in support of the MPO data (Table 1), these differences could not be quantified by histological examination.
Complement activation in ischaemia/reperfusion injury

Figure 2 Transverse sections of gastrocnemius muscle of (a) normal sham-operated muscle from wild-type mice, (b) normal sham-operated muscle from CD46/CD55/CD59 transgenic mice, (c) ischaemic-reperfused muscle from wild-type mice, and (d) ischaemic-reperfused muscle from CD46/CD55/CD59 transgenic mice

Sections were stained with haematoxylin and eosin. Magnification, ×400 (a and d), ×250 (b), and ×300 (c).

DISCUSSION

The finding of improved viability of I/R-injured muscle from CD46/CD55/CD59 transgenic mice compared with that from wild-type mice provides evidence for the involvement of complement activation in the pathophysiology of this injury in skeletal muscle, and is consistent with previous findings in cardiac muscle [5]. The degree of protection provided by complement activation inhibition was, however, less than the dramatic effect achieved earlier in our laboratory by inhibition of mast cell activation [30]. There is known to be a link between the pathways of complement activation and mast cell degranulation [31], although the detailed mechanisms are yet to be elucidated.

The improved tissue viability of I/R-injured muscle from CD46/CD55/CD59 transgenic mice was accompanied by significantly less neutrophil infiltration than that observed in I/R-injured muscle from the corresponding wild-type mice. Stimulation of inflammatory leucocytes (particularly neutrophils and macrophages) by activated complement, in synergy with other inflammatory mediators, may potentiate I/R injury. It has also been observed in the gut that activated complement binds to the vascular endothelium [35,36], causing vascular leakage [4]. When the complement activation inhibitor sCR1 was administered, the ‘permeability index’ was shown to decrease significantly [33]. Interestingly, Chan et al. [19] have recently found that IgM binding to ischaemic skeletal muscle precedes complement activation by several hours. This may have important therapeutic implications when considering anti-inflammatory therapy for I/R injury [27].

The increased binding of complement to the endothelium [35,36] in wild-type mice following I/R may have caused, directly or indirectly, the observed increase in free radical production and XO activity in skeletal muscle. In the transgenic mice used in our experiments, complement-mediated inflammation during I/R was significantly reduced by the presence of the complement activation inhibitors CD46, CD55 and CD59. These molecules presumably reduce the amount of complement adhesion to the endothelium and, in turn, attenuate XO levels and other free radical mechanisms in skeletal muscle, leading to improved muscle viability in the transgenic mice. Hou et al. [37] have postulated that a ‘complement/neutrophil/reactive oxygen species activation feedback’ mechanism operates in inflammatory conditions, including I/R injury. In a clinical study, they found that treatment of patients with antioxidants such as selenium and vitamin E counteracted inflammatory symptoms caused by reactive oxygen species.

In other clinical studies, a strong correlation has been demonstrated between organ dysfunction (e.g. in the heart) and the degree of complement activation measured by circulating blood levels of the anaphylatoxins C3a and C5a [38,39]. Recombinant soluble forms of the three complement activation inhibitors, CD46, CD55 and CD59, have now been produced [40,41] and may be of
some potential benefit if used at the start of reperfusion to stop complement activation before it occurs. Our present study therefore provides a rational basis for the future clinical treatment of I/R and related disorders using complement activation inhibitors, which would specifically target the inflammatory pathways responsible for loss of tissue viability.

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