Role of Sulf1A in Wnt1- and Wnt6-induced growth regulation and myoblast hyper-elongation

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ABSTRACT

Sulf1A expression, which is a characteristic of embryonic muscle, is undetectable in mature muscle fibres and quiescent satellite cells, but is re-activated in vivo upon injury and in vitro following activation of satellite cells. Sulf1A is known to enhance canonical Wnt signalling, and its association with Wnt1-induced satellite cell proliferation in vitro in the present study further confirmed this. However, exogenous Wnt6 decreased satellite cell proliferation but promoted the adoption of a hyper-elongated cell morphology in myoblasts on isolated single fibres in culture. Such Wnt6-induced cellular hyper-elongation and inhibition of proliferation was found to be dependent upon Sulf1A, as treatment with Sulf1A neutralising antibodies abolished both these effects. This indicates that Sulf1A can regulate Wnt6 signalling and cellular differentiation in skeletal muscle.

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1. Introduction

The satellite cells generated during embryonic muscle development persist postnatally as quiescent muscle progenitors under the basal lamina surrounding the muscle fibres [1]. The quiescent muscle progenitors are activated during postnatal growth and in response to muscle injury to repair and regenerate the damaged muscle [1–3]. This resident satellite cell pool is usually sufficient for normal muscle growth and repair. However, in degenerative muscle diseases, satellite cell supply becomes limited due to repeated cell cycles leading to progressive loss of regenerative potential and an ability to maintain muscle mass and function. It is therefore important to understand factors regulating muscle mass since the contributions of different molecular signalling pathways that regulate satellite cell proliferation and differentiation remain poorly defined. The level of satellite cell dependent muscle regeneration relies on the activities of specific growth factors and signalling molecules. These activities are further regulated by the availability of primary and also secondary receptors since many growth factors in addition to their specific primary receptors also require heparan sulphate proteoglycans (HSPGs) as secondary receptors for ligand binding [4]. HSPGs interact with growth factors and receptors through their heparan sulphate (HS) chains, the sulfation status of which can regulate the activities of growth factors requiring HSPG interaction.

HSPG sulfation is not only regulated intracellularly by the activities of Sulfotransferases in the Golgi but is further modified extracellularly by Sulf1 and Sulf2 enzymes, members of the sulfatase gene family related to the lysosomal N-acetyl glucosamine sulfatases [5–7]. Sulf1, the subject of this present study, has been shown to modulate the activities of many growth factors and signalling molecules by regulating the sulfation status of specific HSPGs as it reduces the 6-O-sulfation of heparan sulphate, a component of HSPGs. For example, FGF2 and FGF4 function has been shown to be inhibited by Sulf1 (a full length variant), during chick angiogenesis and in ovarian cells by removing 6-O sulphates from glucosamine residues in heparan sulphate [8,9]. Our recent study [10] also identified a shorter alternatively spliced Sulf1 variant (Sulf1B) in some developing Quail embryonic tissues although full length Sulf1 (Sulf1A) was found to be the major isoform present in developing skeletal muscle. The present study therefore specifically investigated the role of Sulf1A in satellite cell growth that has been shown to enhance canonical Wnt signalling [9,10] although the precise mechanism by which Sulf1A regulates Wnt signalling is not clear but 6-O-desulfation of HSPGs by Sulf1A is believed to modulate the binding affinity of Wnts to heparan sulphate to promote the HSPG-mediated presentation of Wnt ligand to its Frizzled receptor [9]. Wnt signalling has been shown to regulate embryonic muscle development [11] as well as early muscle regeneration [12,13]. To understand the role of Sulf1A and Wnt signalling in muscle growth and regeneration, we used in vitro model of satellite cell activation and growth on dissociated whole skeletal muscle fibres. Dissociated single fibres were grown under normal culture conditions and in the presence of Wnt1 or Wnt6-secreting cells. The quiescent satellite cells

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Fig. 1. Sulf1A expressed in embryonic developing muscle becomes undetectable in adult muscle fibres and quiescent satellite cells but is re-activated in vitro and in vivo regenerating myogenic cells. For example, embryonic myotubes of a 7 day chick limb muscle express both skeletal muscle type myosin heavy chain and Sulf1A as is apparent from the individual and superimposed images (A). Unlike the embryonic muscle, neither adult chickens skeletal muscle fibres (B) or EDL muscle (C) from a 2-week old mouse or their Pax7-positive quiescent satellite cells show any Sulf1A staining by double immunofluorescence procedure. Unlike the skeletal muscle fibres and satellite cells, Sulf1A expression in the same sections is clearly detectable in endothelial cells of blood capillaries (B). Pax7-positive satellite-like cell present in a blood capillary is highlighted by a circle in (B). Pax7-positive satellite-like cell amongst interstitial cells is highlighted by a circle in (C). Myogenic Sulf1 expression, is re-activated in small sized larger unaffected mature muscle fibres in EDL muscle of 4-week old mdx mouse show no Sulf1 expression at either mRNA (D.i, D.ii) or protein (D.iii, D.iv) levels (indicated by asterisks) using in situ hybridisation (D.i, D.ii) or immunofluorescence (D.iii, D.iv) procedures. Similarly, Sulf1A expression is undetectable in Pax7 positive quiescent satellite cells in isolated muscle fibres (E) at time 0 but detectable in most activated Pax7-positive satellite cells at 62 h (F) using double immunofluorescence procedure.

in this study were identified from their expression of a widely used marker of these cells called Pax7, a paired-box tran-fection factor that is expressed in both quiescent and proliferating satellite cells or their progeny required for the maintenance of postnatal skeletal muscle [14,15]. Both Wnt1 and Wnt6 signalling re-activated Sulf1A expression in satellite cells in vitro. While Wnt1 induced a much ear-lier and increased satellite cell proliferation, Wnt6 inhibited satellite cell proliferation by promoting muscle cell differentiation indicated by hyper-elongation of progenitor cells. A reduction in Sulf1A expres-sion by neutralising antibodies, however, reversed the Wnt6 induced inhibition of satellite cell proliferation as well as myoblast hyper-elongation.

2. Materials and methods

2.1. In situ hybridisation procedure

Extensor digitorum longus (EDL) muscles of control and mdx mice were fixed overnight in 4% paraformaldehyde (PFA) at 4 °C before Paraffin embedding and sectioning at 6 or 10 μm thickness. A digoxigenin-labelled 292 bp riboprobe to mouse Sulf1A (1686–1978 bp) was used to analyse mRNA expression using in situ hybridisation procedure described by Moorman et al. [16].

Ethical statement: Muscles for all experiments in this study were removed from animals sacrificed following the Home Office (UK) licence Schedule 1 procedure in accordance with the Animals (Scientific Procedures) Act 1986 and with approval from the Royal Veterinary College ethical review process.

2.2. Cell culture and muscle fibre isolation

The EDL muscles were carefully dissected from 8 to 10 week old CD mice (killed using Schedule 1 procedure) and muscle fibres dissociated in 0.12% Type 1 Collagenase (Sigma)/DMEM [13,17]. Single fibres were cultured in 24-well plates with 12–15 fibres/well in 0.5 ml/well culture medium (10% FCS/0.5% Chick embryo extract/DMEM). The single muscle fibres were incubated at 37 °C, 5% CO2 before fixation in 4% PFA for 15 min at different time intervals indicated in Section 3. Some muscle fibres were also fixed at time 0 to investigate quiescent satellite cell phenotype. For satellite cell growth in the presence of Wnt1 and Wnt6, mouse NIH-3T3 Wnt1 (provided by Dr. A. Kispert), Wnt6 (provided by Dr. S. Vainio) and Lac-Z-transfected control cells were grown in 24-well plates to 50–60% confluency before 12–15 dissociated muscle fibres were added and co-cultured for 24, 48 or 72 h before fixation in 4% PFA. In some experiments, 100 ng or 300 ng/ml neutralising antibody to Sulf1A [17] was added to the culture medium at time 0 and the satellite cells on single fibres cultured for 72 h before fixation and staining with different antibodies.

2.3. Immunocytochemical procedure

Different polyclonal and monoclonal antibodies were used to study the expression patterns of Sulf1A, Pax7 and MyoD. A Sulf1A specific rabbit polyclonal antibody (B) raised to an 18 amino acid peptide in the hydrophilic domain was used for most analyses [10] although another Sulf1 antibody (C) raised to a 20 amino acid peptide present in the C-terminal domain of Sulf1 was also used in some
vitr**o** and in **v**ivo regenerating myotubes mature muscle fibres and quiescent satellite cells but is re-activated in

3. Results

was performed using student t-test, regarding p < 0.05 as statistically significant.

fibre**s** that did not stain for Sulf1A was confirmed from their positive staining for Pax7, a ubiquitously expressed marker of satellite cells.

Sulf1A and MyoD, characteristic markers of early embryonic muscle, are undetectable in Pax7-positive quiescent satellite cells but are both re-expressed in activated satellite cells (Figs. 1 and 2). Satellite cell development therefore was further investigated in the presence of Lac-Z transfected control and Wnt1 and Wnt6 secreting cells using Sulf1A and MyoD as markers of satellite cell activation and growth. MyoD and Sulf1A expression was apparent in both control and Wnt1 or Wnt6 treated satellite cells. Compared with the control cultures, the level of satellite cell proliferation (measured as an increase in the number of cells/cluster) in the presence of Wnt1 secreting cells, however, was markedly increased (Figs. 2 and 4) as also demonstrated by Otto et al. [13]. The onset of satellite cell division in the presence of Wnt1 was found to be much earlier when compared with control growth conditions. For example, while satellite cells in normal growth medium or in the presence of Lac-Z transfected cells are rarely observed undergoing cell division before 24 h, satellite cell proliferation in the presence of Wnt1 was frequently apparent at 12–13 h in vitro (Fig. 2).

The in vitro culture of isolated single muscle fibres in the presence of Wnt6 secreting cells also led to a marked change in satellite cell growth. However, unlike Wnt1 cells showing earlier and increased level of satellite cell proliferation, Wnt6 exposure inhibited satellite cell proliferation measured as number of cells/cluster (Figs. 2–4). Reduced satellite cell proliferation in the presence of Wnt6 cells was observed throughout the 72 h culture time period (Figs. 2–4). The other marked feature of Wnt6 on satellite cell growth was its very distinctive effect on myogenic cell morphology (Figs. 2 and 3). Unlike satellite cells grown in the presence of Wnt1 secreting cells and normal culture medium, satellite cell generated myogenic cells in the presence of Wnt6 were usually hyper-elongated (Figs. 2–4). The onset of such morphological change often became apparent within 24 h in vitro, initially adopting a spindle shape resembling myoblast morphology, although the degree of elongation increased with time.
and appeared to be related to the distance between neighbouring myogenic cells. For example, the elongation index (obtained by the maximal myogenic cell length divided by the maximum cell width at the centre of the nucleus) for Wnt6 exposed satellite cells at 24, 48 and 72 h had significantly increased when compared with satellite cells grown in the presence of Wnt1 and Lac-Z control or normal growth medium (Fig. 4). Unlike Wnt6, the elongation index for the satellite cells grown in the presence of Wnt1 and control cultures over 24–72 h time period showed only a minor change in morphology (Fig. 4). Sulf1A expression in Wnt6-induced satellite cell extensions was not uniform but regularly punctuated (indicated by white lines in Fig. 2) until it reached or contacted the extension on its nearest neighbouring myogenic cell with increased expression level (an arrow in Fig. 2).

3.3. **Sulf1A inhibition by neutralising antibodies reverses Wnt6-induced inhibition of satellite cell proliferation and myoblast hyper-elongation**

Since exogenous Wnt6 inhibited satellite cell proliferation and induced marked changes in cellular morphology despite endogenous Sulf1A being present in these cells, we further investigated the function of Sulf1A by reducing Sulf1A levels in satellite cells cultured in the presence of Wnt6 secreting cells. Sulf1A reduction by 100 ng/ml antibody concentration did not only restore cell proliferation but also reversed the cell shape change leading to near normal cell morphology after 72 h in vitro growth (Fig. 3) as indicated by the increased satellite cell number/cluster and reduction in myogenic hyper-elongation index (Fig. 4). A further increase in neutralising Sulf1A antibody concentration to 300 ng/ml initially enhanced proliferation but was followed by apparent apoptosis and was therefore avoided in later experiments by using only 100 ng/ml in the present study.

4. **Discussion**

Our previous study demonstrated the importance of Sulf1A in embryonic muscle development since Sulf1A inhibition by antisense oligos led to down-regulation of MyoD transcription factor, an early marker of myogenesis [20]. Unlike the embryonic muscle, Sulf1A was undetectable in postnatal mature muscle fibres as well as Pax7 positive quiescent satellite cells associated with post-natal skeletal muscle fibres. Sulf1A expression, however, was re-activated in satellite cells on isolated muscle fibres cultured in vitro and in vivo regenerating myotubes of mouse muscles, thus re-capitulating early muscle development where it may regulate the activities of specific growth factors or signalling molecules. Once activated, Sulf1A expression in vitro continued in most satellite cells up to the 72 h time period investigated in this study. During this early phase of myogenesis, Sulf1A could regulate multiple growth factor signalling pathways including Wnt signalling since canonical Wnt signalling has been shown to be enhanced by Sulf1A [9,10,20,21].

In addition to increased satellite cell proliferation, in vitro exposure to Wnt1 induced earlier onset of satellite cell division. We did not, however, experimentally alter the Sulf1A levels in the presence of Wnt1 but Sulf1A was expressed in all satellite cells during Wnt1 exposure. The co-expression of Sulf1A with Wnt1 could enhance canonical Wnt signalling as shown for other cells in earlier studies [9,10,20,21]. Unlike Wnt1, Wnt6 did not only show much reduced satellite cell proliferation but also a marked change in cell morphology, indicating possible onset of differentiation or its role in cell fusion. For example, while satellite cells grown in the presence of Wnt1 maintained the regular cell size and shape observed in satellite cells grown under normal culture conditions, satellite cells grown in the presence of Wnt6 showed hyper-elongation of myoblasts demonstrating that different Wnt signalling pathways do not only selectively regulate satellite cell proliferation but also myogenic differentiation and possibly cell fusion distinctly. Sulf1A inhibition achieved by the addition of Sulf1A neutralising antibodies [17], however, overcame Wnt6-induced inhibition of satellite cell proliferation as well as morphological change indicative of onset of differentiation. The level of satellite cell proliferation following Sulf1A inhibition had increased to that seen in control culture levels. Sulf1A is known to enhance canonical Wnt signalling whereas the present study demonstrates that Sulf1A also plays a role in Wnt6 signalling as indicated by restoring near normal satellite cell proliferation and morphology following Sulf1A inhibition. This may be due to Sulf1A’s distinct role in Wnt6 signalling since Wnt6 has the capacity to act via both canonical and non-canonical pathways. Further studies, however, would be required to confirm the role of Sulf1A in Wnt6 regulation by not only Sulf1A inhibition but also using purified Sulf1A protein. While the expression and role of canonical Wnt signalling in muscle development is well established, little is known about either the expression or role of Wnt6 in muscle growth. Otto et al. did not observe Wnt6 expression in activated satellite cells at 24 h in vitro [13] but considering its inhibitory role in satellite cell proliferation, Wnt6 expression may need to be investigated during later stages of satellite cell growth to understand its role in myoblast cell fusion and differentiation.

The opposing roles of Wnt1 and Wnt6 on satellite cell proliferation and cell morphology demonstrate distinct functions of different Wnt signalling pathways in the regulation of muscle mass. Muscle growth therefore could be regulated by competing Wnt signalling pathways, with canonical signals promoting growth through increased proliferation while non-canonical signals favouring differentiation. This study demonstrated that Sulf1A is present in activated satellite cells and its inhibition can reverse Wnt6-induced satellite cell behaviour, presumably by blocking non-canonical actions of Wnt6. During muscle regeneration, the function of Sulf1A therefore may be to regulate the levels of different Wnt signalling pathways so that satellite cell proliferation and differentiation occur in a co-ordinated manner to regulate muscle mass. While Wnt6 signalling has been reported to inhibit myocardial growth in some other tissues such as developing heart [22], this is a first study to highlight the role of Wnt6 signalling pathway in skeletal muscle mass regulation by not only preventing satellite cell proliferation but also by preventing myogenic cell size growth.

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