Direct cell-cell communication through gap junctions during early skeletal muscle development and regeneration in the rat

Summary of PhD Thesis

Anikó Görbe MD

Department of Biochemistry, Faculty of Medicine

University of Szeged, Hungary

2007

INTRODUCTION

Skeletal myogenesis is a complex process that begins during somitogenesis in the embryo and continues through postnatal development. Muscles are assembled by fusion of individual postmitotic myoblasts to form multinucleated syncytial myotubes. Skeletal muscle development implies a near-synchronous switch from independent proliferation towards aligned differentiation across the entire population of myoblasts, and several lines of evidence implicate gap-junctional communication as coordinating factor in this switch.

Embryonic development of muscles starts with the proliferation of muscle precursor cells, which are in a stem cell like stage, with the potential to become muscle cells. This early stage is controlled by myogenic transcription factors including Myf-5, and MyoD, which are responsible for myoblast determination. Differentiation and aligment of myoblasts are regulated by myogenin, Mrf-4 and p21waf1/Cip1 a cyclin-dependent kinase inhibitor. Activation of genes of muscle specific and cell adhesion molecules and interactions between the pathways of myogenic regulation and cell cycle control eventually result in orderly aligned myoblasts prepared for syncytial fusion.

Vertebrate skeletal muscle has ability to regenerate from quiescent progenitors called satellite cells that lie between the sarcolemma and basal lamina of muscle fibres. These satellite cells are activated by mitogens released from the injured fibres and they use the same differentiation programme until early myotube formation either in vivo or in culture offering a superb model for studying myogenesis.

The large scale fusion process takes place within a narrow time frame which suggests that there is a precise coordination of cell cycle exit of aligned myoblasts. Direct cell–cell communication through gap junctions, which has been detected in differentiating myogenic cells, but not in mature

myofibres of vertebrates, could contribute to the coordination of this process.

Gap junction channels are formed from docking of hemichannels (connexons) in opposing cell membranes of adjacent cells. Each connexon is created from 6 connexin (Cx) protein molecules. The Cxs are in a multigene family with 21 members in the human genome, commonly named according to their molecular weight (e.g. Cx43 is 43 kDa). Gap junction channels may provide direct pathway for small molecules (<1kD), metabolites and second messengers such as IP_3 and cAMP, and ions e.g. Ca^{2+}, to rapidly pass between adjacent cells. Cell membrane associated non-aligned hemichannels are also capable of releasing active metabolites into the extracellular space and the Cxs may even have channel unrelated regulatory roles. Gap junction coupling allows the propagation of signals through extensive cell networks or restricted tissue compartments and junctions influences a wide range of cellular and tissue activities, including regulation of growth, differentiation, and developmental signalling.

Gap junctions and connexins have been found transiently in embryonic and newborn skeletal muscles of the chick, mouse and the rat, though their roles are still to be elucidated. Gap junctions have also been detected in myogenic cell cultures during the prefusion period and their disappearance was observed at terminal muscle differentiation. Cxs, including Cx36, 39, 40, 42, 43 and 45, shows a species and developmental stage specific expression. Application of channel blockers, such as octanol and β-glycerrhetinic acid, or the inducible deletion of Cx43 gene interferes with early myogenic differentiation resulting in reduced expression of myogenic factors MyoD, myogenin and MRF4, and/ or inferior or delayed myoblast fusion Furthermore, transfection of rhabdomyosarcoma cells with Cx43 cDNA has been published to induce cell differentiation. Most of these data, however, have been obtained in transformed cell lines, which may differ significantly from primary myogenic cells. No comprehensive study has been performed in vivo or in primary cultures to reveal the regulation of gap junction Cx expression in the course of myogenic progenitor cells differentiating into multinucleate myofibers.

AIM

The aim of this study was to determine the spatio-temporal regulation of gap junction connexin expression and function during early myogenic differentiation using both *in vivo* and *in vitro* rat models. Ultrastructural, immunomorphological, and functional dye transfer techniques were utilized in combination with laser scanning microscopy, quantitative image analysis and gene transfection studies.

1) Notexin induced muscle regeneration model recapitulating *in vivo* myogenesis was used to study the spatio-temporal expression of gap junction Cx isoforms during myoblast differentiation and fusion in correlation with factors of cell proliferation (Ki67) and cell cycle control (p21waf1/Cip1 p27kip1).

2) Primary myoblast cultures set up from satellite cells of the same species used for the regeneration model were used to study the temporal correlation of Cx expression and functional dye coupling through Cx43 gap junctions, with particular attention to the prefusion period, using quantitative image data analysis.

3) Primary myoblast cultures also, were used to manipulate Cx43 expression and function using gene transfection with wild-type or dominant-negative eGFP (enhanced green protein)-Cx43 vector constructs, as well as an eGFP-only control (wtCx43, dnCx43 or GFP-only). Functional changes of up- and downregulated gap junction coupling were correlated with myoblast differentiation and fusion.

MATERIALS AND METHODS

Notexin induced regeneration in vivo

Muscle regeneration was induced in musculus soleus of adult Wistar rats using notexin, the venom of a mainland tiger snake (Notechis scutatus scutatus). Muscle samples were excised on days 1,2,3,4,5 and 7 after notexin administration, and tissues were processed for morphological and immunohistochemical analysis. Morphological analysis was based on semithin resin sections stained with methylene blue and basic fuchsine and on ultrathin sections of 80-100 nm thin cut for electron microscopy as well.

Isolation and culturing newborn rat myoblasts

Muscle samples were originated from the hind limbs of newborn rats and treated with 0.25% trypsin. Digested cell suspension was centrifuged twice, and the pellets resuspended in complete Dulbecco's MEM (DMEM) growth medium containing 10% fetal calf serum (FCS), 10% horse serum and 200 μg/ml Gentamycin. Cells were grown on coverslips and examined daily to count transfected cells, or removed for immunostaining or functional dye-transfer testing.

Immunostaining for single and double antigens

Paraffin sections were immunostained for desmin, $p21^{waf1/Cip1}$, -$p27^{kip1}$, Ki67 and visualised by immunoperoxidase staining.

Cryostat sections were cut from the frozen regenerated muscle samples, and treated with the following antibodies: anti-desmin, muscle specific actin, myo-D1 and different mono and polyclonal anti-connexin antibodies (Cx26, 32, 37, 40, 43, 45). Adherent myoblast cultures grown on coverslips were stained with the same set of primary antibodies in the same way as frozen sections.

Sections stained for immunofluorescence were examined with a Leica TCS SP confocal laser-scanning microscope using 1024x1024 pixels resolution either in single or dual channel (FITC/TRITC) modes.

Quantitative image analysis of Cx43 and cell cycle associated proteins

Digital single channels images of regenerated muscle samples stained either for Cx43 and cell cycle associated proteins were transformed into 8 bit black-and-white format and were analyzed using the ImageJ 1.29x software Connexin43 protein expression in myoblast cultures was also analyzed quantitatively in the same way during the differentiation process.

Transfection of adherent myoblasts

Two days after plating, adherent primary myoblast cultures were transfected with pIRES2-eGFP vectors containing sequences coding for eGFP alone, eGFP and wild-type Cx43 (wtCx43), or eGFP and a dominant-negative construct. Cultures were monitored for 1–4 days after tranfection (3–6 day-old cultures) using a fluorescence microscope.

Dye transfer assays

Samples of myoblast cultures grown on coverslips were taken out daily between day-1 and day-5 of normal cultures or 3-6 days after transfection. Dye coupling of myoblasts through gap junctions was studied with two microinjection assays. Either the gap junction permeant Lucifer Yellow alone or the combination of the gap junction impermeant Dextran-Fluorescein and the junction permeant Cascade Blue analogue were used. Microelectrodes were introduced into the cytoplasm of myoblasts under visual control and small pulses of negative current were used to ionophorese the dye(s) into

the cells. The spread of fluorescent tracers was visualized under the fluorescent microscope with CCD camera.

RESULTS

Gap junctions in notexin treated skeletal muscle

In intact skeletal muscle and in notexin-induced muscle regeneration six connexin isotypes were tested (26, -32, -37, -40, -43 and -Cx45), and only Cx43 showed reliable immunoreaction on desmin positive myogenic cells as compared to control samples.

In early regeneration just only few myoblasts were detected and parcially all were in the cell cycle as characterized by strong nuclear Ki67 staining and none were positive for $p21^{waf1}$ and $p27^{kip1}$ (cyclin-dependent kinase inhibitors) reactions. Connexin43 immunostaining was limited at this stage and it was also confined to the myoblasts along pre-existing muscle fibers.

Two days later, desmin positive myoblast were apparent at large numbers and gathered along the pre-existing muscle fibers and produced significant amount of punctuate Cx43 staining. Only weak $p21^{waf1}$ and $p27^{kip1}$ nuclear staining could be detected in less than 30% of them. At the same time, nearly 90% of myoblasts strongly expressed the proliferation associated Ki67 nuclear protein. Double immunolabeling co-localized Cx43 and desmin in myogenic cells.

Three days after notexin administration large numbers of myoblasts were aligned in close membrane apposition along the basal lamina, which was accompanied with the highest amount of Cx43 detected produced mainly by myoblasts. Myoblasts showed massive expression of $p21^{waf1/Cip1}$ and $p27^{kip1}$ while lacking the proliferation marker Ki67, which indicated their synchronized exit from the cell cycle.

Fourth day of regeneration myoblast alignment was clearly visible with most of them completed fusion and newly formed myotubes started forming bundles of contractile elements. Connexin43 protein expression rapidly declined with the progression of myoblast fusion into syncytial myotubes. Cell cycle control proteins were detected in large numbers of myonuclei. By day-7, newly formed muscle fibers showed conspicuous bundles of contractile elements and the regeneration process was completed between days 21-28.

Gap junctions in differentiating non-manipulated primary myoblast culture

Similarly to our in vitro model, myoblasts were identified with desmin immunostaining to exclude non-muscle cells from the study. Same set of Cx isotypes were tested in rat primary myoblasts and only Cx43 was detected, which is line with our findings in regenerating of skeletal muscle. Over 90% of cultured cells were desmin positive.

In day-1 cultures, myoblasts have round or slightly elongated shape and started to express Cx43 protein. Double immunolabeling for Cx43 and desmin confirmed cell membrane-bound and cytoplasmic Cx43 not only in cells of physical contacts with each other, but also in non-interacting myoblasts.

In day-2 cultures spindle shaped myoblasts were linked to their neighbors and double labeling proved Cx43 and desmin co-localization in them. Cx43 protein expression was greatly upregulated and detected as dotlike signal along myoblast membranes and massive granular immunostaining in their paranuclear region reflecting extensive production and assembly of gap junction channels. By day-3, most contacting myoblasts were aligned and membrane associated Cx43 particles increased substantially, while the cytoplasmic fraction significantly reduced. The peak of Cx43 expression was detected on the 2^{nd} and 3^{rd} day of regeneration.

By day-4, numerous multinucleate myotubes were formed. Connexin43 immunostaining was restricted to mononucleate

myoblasts and only very few signals were detected in myotubes. By day-7, large myotubes and increasing numbers of dying myogenic cells were seen.

Gap junctional coupling was tested in primary myoblast cultures using Lucifer Yellow fluorescent dye, which was microinjected into single cells that were in contact with their neighbors. In sparse day-1 cultures, the communicating cells were coupled to 1-2 neighbors most they were in contact with. Significant elevation in cell coupling was found in day-2 cultures, where the dye frequently passed into several cells in the 2nd and 3rd order from the injected myoblast. Day-3 cultures were still coupled, but less myoblast were involved despite of the peak of Cx43. The coupling frequency declined further by day-4 and at later stages upon progression of myoblast fusion and the appearance of increased numbers of myotubes.

Gap junctions in manipulated primary myoblast cultures

To further determine the contribution of Cx43-mediated gap-junctional communication to myoblast proliferation, differentiation and fusion into myotubes, we manipulated the expression of Cx43 in myoblast cultures. Transfection of adherent myoblast cultures on the 2nd day of culture, prior to the normal peak of Cx43 expression, was performed with pIRES2-eGFP, either alone (GFP-only) to demonstrate the consistency of transfection. We analysed 12 parallel cultures, all treated with pIRES2-eGFP construct, and the transfection was found to be consistent in parallel cultures.

Our main aim was to examine the effects on myoblast numbers and myotube formation with modifying the connexin content of the myoblasts, either by overexpressing the wild-type Cx43 sequence or by expressing a well-characterized dominant-negative version that disrupts junctional communication. Four coverslips treated with each construct were examined on each day after transfection. Cells over-expressing wtCx43 were seen to form as many GFP$^+$ myotubes as cells transfected with GFP only, and appeared to maintain them in the GFP$^+$ state for longer. In contrast, cells transfected with the communication-impairing dnCx43 construct increased their numbers much more than those in either of the other groups during the pre-fusion period, up to two days after transfection, and most of them remained in a potentially proliferative, unfused state thereafter. Such cells were found in large numbers as GFP$^+$ mononucleate cells throughout the experiment, and were significantly retarded in myotube formation.

Dye coupling tests combining the gap junction permeable Cascade Blue analogue and the channel-impermeable Dextran-fluorescein (can pass only when cytoplasmic continuities are present) was applied in non-manipulated primary myoblast cultures first, and resulted in similar data to those gained with the Lucifer Yellow assay. In summary, wtCx43 cells, which would be expected to have more functional gap junctions than the other groups, did indeed show higher levels of junctional coupling; but they also showed cytoplasmic coupling both earlier and more extensively than the other groups, consistent with precocious entry into the early stages of cell fusion. Conversely, in dnCx43 cells, our coupling data not only showed that the presence of the dominant-negative connexin reduced gap-junctional communication at all stages, as expected, but also that its presence almost eliminated cytoplasmic coupling, reinforcing our morphological observation that dnCx43 cells were retarded in cell fusion.

DISCUSSION

We tested muscle sections and myoblast cultures of rats for six connexins and we found only Cx43, the isotype, which was detected by most research groups in vertebrate myogenic cells either in tissues or in cell cultures. Immunostaining for desmin and Cx43 showed that gap junctions were expressed by myogenic cells, which direct evidence was missing from earlier works. In the chick, Cx36 and -42 and -45 were found in the myotome and/or the dermomyotome of somites and Cx43 in the pectoral muscle of the embryo. In the mouse, Cx39 and Cx40 were described in embryonic myotubes, while Cx43 and -45 were detected in regeneration, which results suggested that there might be a switch between Cx isotypes during muscle development, or Cx gene expression may differ between mice and rats. These data suggest that more than one Cx isotype is involved during early muscle differentiation in vertebrates, but Cx43 channels seems to be dominating.

Formation of gap junctions is a ubiquitous feature of cells devoted to multicellular functions and several studies have detected gap junction connexins and/or cell coupling in myogenic cells before fusion and downregulation of Cxs in multinucleate myotubes. However, the way, as gap-junctional coupling influences muscle development, myoblast proliferation, differentiation and fusion still needed further clarification.

In this study, we provide in vivo and in vitro evidence on the transient expression and developmental regulation of gap junction protein in differentiating myoblasts preceding fusion by differentiating pre- and postmitotic stages. Cx43 appeared early in proliferating single myogenic cells, followed by a progressive upregulation in the cytoplasmic and cell-membrane domains of interacting myoblasts until fusion and then by a rapid fall in multinucleate myotubes. To participate in the large-scale fusion within a short-time-frame as it happens normally, a multitude of proliferating myoblasts must leave the cell cycle synchronously and escape apoptosis. In regeneration, elevated production of Cx43 channels was accompanied by the nuclear expression of cyclin dependent kinase inhibitor proteins and the complete loss of the proliferation associated protein suggesting a synchronized exit of cell cycle before fusion and a potential role of gap junction coupling in regulating these processes. In myoblast cultures, the temporal regulation of dye coupling was correlated with connexin expression. Intracellular injection and cell-to-cell spreading of a gap-junction-permeant dye such as Lucifer Yellow is an established technique for assessing functional gap-junction coupling. Most extensive dye transfer was found in sparse, day 2 cultures, which result was confirmed by combining a gap junction permeant Cascade Blue, mixed with a fluorescent dextran, to distinguish junctional coupling from dye spread through cytoplasmic continuities.

In searching for further functional evidenve, Cx43 expression of early myoblasts was manipulated by transfecting them with wild-type or dominant-negative eGFP-Cx43 constructs, or with an eGFP-only control. Myoblasts transfected with wtCx43 showed more gap-junctional coupling than GFP-only controls, began fusion sooner and formed more myotubes. DnCx43 transfected cultures remained proliferative for longer than either GFP-only or wtCx43 myoblasts, showed less coupling, and underwent little fusion into myotubes.

A potential role for Cx43 channels in skeletal myogenesis as they promote rapid fusion of myoblasts, would fit into the generally proposed functions of gap junctions e.g. in controlling cell growth for promoting a more differentiated phenotype and/or in coordinating cell functions in a network of cells. We observed precocious cytoplasmic coupling at pre-fusional stages when wtCx43 is overexpressed, and with the strong fusion-delaying effects of dnCx43.

Both unpaired connexons and aligned gap junctions are thought to form important pathways for regulating intracellular Ca^{2+} levels which play a major part in early myogenic

differentiation and cell fusion. Besides Ca level regulation, Cx43 expression and function in differentiating myoblasts could influence signaling pathways, as transient elevation of cAMP, a known positive regulator of Cx43 expression and channel permeability is detected during early myoblast differentiation. However, all of these possibilities may require further investigations.

New observations in our studies:

1. The progressive upregulation of Cx43 gap junction protein in prefusion myoblasts by dissecting pre- and postmitotic stages, and the rapid decline of Cx43 expression after fusion during skeletal muscle differentiation *in vivo* using a rat regeneration model.

2. The spatio-temporal expression of gap junction connexins correlated with the progression of cell cycle control, as the significant upregulation of Cx43 gap junctions in aligned myoblasts preceding fusion was accompanied by the nuclear expression of cyclin-dependent kinase inhibitors $p21^{waf1/Cip1}$ and $p27^{kip1}$ and the complete loss of Ki67 protein.

3. Rat cultured myoblasts showed similar temporal regulation of Cx43 expression and phenotypic maturation to those regenerating *in vivo*. However in prefusion myoblasts we detected extensive GJ coupling between the sparse and proliferating myoblasts and a reduced communication between the aligned, postmitotic cells. This finding functionally supported that that we found in regeneration, namely GJIC has a role in controlling myoblast cell cycle.

4. Myoblasts transfected with wtCx43 showed more gap-junctional coupling than the GFP-only controls, began fusion sooner as judged by the incidence of cytoplasmic coupling, and formed more myotubes. However, myoblasts transfected with dnCx43 remained proliferative for longer than either GFP-only or wtCx43 myoblasts, showed less coupling, and underwent little fusion into myotubes. These results further confirmed the role of gap junction direct cell-cell communication in controlling and probably synchronising myoblast cell cycle for a coordinated syncytial fusion.

AKNOWLEDGEMENTS

These studies were supported by grants from the Hungarian Ministry of Education and Royal Society (UK).

I greatly acknowledge to Prof. László Dux and David L. Becker for providing me the excellent opportunities for working in a highly inspiring professional environment both at the Department of Biochemistry at University of Szeged and in the Confocal Unit of the Centre of Cell Dinamics at University College London (UCL).

I am greatly indebted to my tutor, Dr Tibor Krenács, for his scientific guidance, encouragement and support.

I am very grateful to Mrs. Elizabet Balazshazi, Zsuzsa Lajtos, Zita Felhő, Anikó Sarró, Mária Labdy and Katalin Danyi (University of Szeged) and to Mr. Daniel Ciantar (UCL) for skilfull technical assistance.

I am very much obliged to my colleagues for always being ready to help me.

I am especially grateful to my mother and father for their continuous help and endless support and also to my family and friends for encouraging me to accomplish this work.

PUBLICATIONS

Full papers directly related to the subject of the thesis:

I. Aniko Gorbe, David L Becker, Laszlo Dux, Eva Stelkovics, Laszlo Krenacs, Eniko Bagdi, Tibor Krenacs
Transient upregulation of connexin43 gap junctions and synchronized cell cycle control precede myoblast fusion in regenerating skeletal muscle *in vivo*
Histochem Cell Biol (2005) 123:573-83 IF: 2.239

II. Aniko Gorbe, David L Becker, Laszlo Dux, Laszlo Krenacs, Tibor Krenacs
In differentiating prefusion myoblasts connexin43 gap junction coupling is upregulated before myoblast alignment then reduced in post-mitotic cells.
Histochem Cell Biol (2006) 125:705-16 IF: 2.239

III. Aniko Gorbe, Tibor Krenacs, Jeremy Cook, David L Becker
Myoblast proliferation and syncytial fusion both depend on connexin43 function in transfected skeletal muscle primary cultures.
Exp Cell Res (2007) 313:1135-48 IF: 4.418