A synthetic glycosaminoglycan mimetic (RGTA) modifies natural glycosaminoglycan species during myogenesis

Isabelle Barbosa[1], Christophe Morin[1], Stephanie Garcia[1], Arlette Duchesnay[1], Mustapha Oudghir[2], Guido Jenniskens[3], Hua-Quan Miao[4], Scott Guimond[5], Gilles Carpentier[1], José Cebrian[1], Jean-Pierre Caruelle[1], Toin van Kuppevelt[6], Jeremy Turnbull[5], Isabelle Martelly[1,*] and Dulce Papy-Garcia[1]

[1]Laboratoire CRRET, CNRS UMR 7149, Université Paris 12-Val de Marne, 61 Avenue du Général de Gaulle, 94010 Créteil CEDEX, France
[2]Faculty of Science Semlalia, University Cadi Ayyat, BP 2390, Marrakech, Morocco
[3]Division of Bioengineering and Environmental Health, Massachusetts Institute of technology, Cambridge, MA 02139, USA
[4]ImClone Systems Incorporated, New York, NY 10014, USA
[5]School of Biological Sciences, University of Liverpool, Liverpool, L69 7ZB, UK
[6]Department of Biochemistry194, University Medical Centre, NCMLS, PO Box 9101, 6500 HB Nijmegen, The Netherlands
*Author for correspondence (e-mail: martelly@univ-paris12.fr)

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Summary

Crucial events in myogenesis rely on the highly regulated spatiotemporal distribution of cell surface heparan sulfate proteoglycans to which are associated growth factors, thus creating a specific microenvironment around muscle cells. Most growth factors involved in control of myoblast growth and differentiation are stored in the extracellular matrix through interaction with specific sequences of glycosaminoglycan oligosaccharides, mainly heparan sulfate (HS). Different HS subspecies revealed by specific antibodies, have been shown to provide spatiotemporal regulation during muscle development. We have previously shown that glycosaminoglycan (GAG) mimetics called RGTA (ReGeneraTing Agent), stimulate muscle precursor cell growth and differentiation. These data suggest an important role of GAGs during myogenesis; however, little is yet known about the different species of GAGs synthesized during myogenesis and their metabolic regulation. We therefore quantified GAGs during myogenesis of C2.7 cells and show that the composition of GAG species was modified during myogenic differentiation. In particular, HS levels were increased during this process. In addition, the GAG mimetic RGTA, which stimulated both growth and differentiation of C2.7 cells, increased the total amount of GAG produced by these cells without significantly altering their rate of sulfation. RGTA treatment further enhanced HS levels and changed its subspecies composition. Although mRNA levels of the enzymes involved in HS biosynthesis were almost unchanged during myogenic differentiation, heparanase mRNA levels decreased. RGTA did not markedly alter these levels. Here we show that the effects of RGTA on myoblast growth and differentiation are in part mediated through an alteration of GAG species and provide an important insight into the role of these molecules in normal or pathologic myogenic processes.

Key words: Glycosaminoglycans, Myoblast, RGTA

Introduction

The basal lamina surrounding muscle fibres fulfils many developmental and physiological roles (Sanes, 2003; Sanes et al., 1990; Sanes et al., 1986). The basal lamina networks are interconnected with resident and cell surface proteins and proteoglycans (PG). These PGs consist of a core protein to which glycosaminoglycan (GAG) moieties are covalently linked. Heparan sulfate proteoglycans (HSPG) constitute the major portion of proteoglycans of the basal lamina of skeletal muscle cell surfaces (Brandan et al., 1996; Brandan et al., 1991; Cornelison et al., 2001; Larrain et al., 1997). Highly regulated spatiotemporal distribution of basal lamina components and of cell surface HSPGs such as collagen, agrin, perlecan, glypicans or syndecans (Sanes et al., 1990) create a specific microenvironment around muscle cells that act as a determinant for the events of myogenesis in particular by regulating bioavailability of growth factors (Larrain et al., 1997; Larrain et al., 1998). A growing body of evidence indicates that most growth factors involved in the control of cellular growth and differentiation can be stored in the extracellular matrix through specific interaction with GAGs, especially heparan sulfate (HS). Heparin or HS stabilizes and protects growth factors from degradation (Sommer and Rifkin, 1989) and may even potentiate their activity (Lyon and Gallagher, 1998; Rahmoune et al., 1998; Rapraeger, 2002). From these binding sites, the growth factors traditionally designated as heparin binding growth factors (HBGF), can be released and accomplish cellular activating functions.

The control of bioavailability of HBGF may in part be mediated by an affinity of these factors for specific sequences of the sugar moiety of proteoheparan sulfates. In particular, the pattern of sulfation regulates the affinity of growth factors to

HS (Fernig et al., 2000; Ford-Perriss et al., 2002; Kreuger et al., 1999; Kreuger et al., 2001). Interestingly, it has been demonstrated, using phage display antibodies, that HSs form a family of molecules that present a spatiotemporal regulation in differentiating skeletal muscle cells, thus suggesting a subtle regulating role of these molecules (Dennissen et al., 2002; Jenniskens et al., 2000; Jenniskens et al., 2002). Indeed, several growth factor binding oligosaccharides sequences have been established (Allen et al., 2001; Kreuger et al., 2001; Olwin and Rapraeger, 1992; Turnbull et al., 1992). They govern the activity of growth factors on cells, as has been shown for instance for fibroblast growth factor 2 (FGF2) (Berry et al., 2003; Rapraeger et al., 1991).

We have used substituted dextran polymers that mimic GAGs on the basis of their ability to interact with and to protect several HBGFs against proteolysis (Meddahi et al., 1995; Meddahi et al., 1996). These GAG mimetics were shown to enhance tissue repair in various in vivo models including skin (Meddahi et al., 1996), bone (Blanquaert et al., 1995) or colon (Meddahi et al., 2002). They highly stimulate and improve regeneration of denervated and crushed skeletal muscles (Aamiri et al., 1995; Gautron et al., 1995; Zimowska et al., 2001) as well as prevent most of the damage resulting from acute skeletal or cardiac muscle ischemia (Desgranges et al., 1999; Zakine et al., 2003). Therefore, these dextran polymers were called RGTA (for ReGeneraTing Agent). Experiments performed with satellite cells (also called muscle precursor cells) in primary cultures have shown that these GAG mimetics stimulate satellite cell growth and differentiation (Papy-Garcia et al., 2002; Stockholm et al., 1999).

Taken together, these results suggest that the GAG moiety of certain proteoglycans might regulate the differentiation process of myoblasts into myotubes. However, little is known about the GAG composition of myoblasts and myotubes. Chondroitin sulfate (CS) and HS synthesis are greatly increased in *mdx* satellite cells compared to normal muscle satellite cells (Alvarez et al., 2002; Crisona et al., 1998). The reduction of GAG sulfation by chlorate treatment of C2 myoblasts reduces their growth (Papy-Garcia et al., 2002) and differentiation capacities (Melo et al., 1996).

These results prompted us to study changes in GAG levels during myoblast differentiation in vitro and to identify their species, using the C2.7 cell line. We also investigated whether a specific RGTA, named RGD120 and structurally related to heparin, would alter the content of the GAG species balance in relation to its effect on myoblast growth and differentiation.

Materials and Methods

Materials

The RGTA used in this study, RGD120, was obtained from OTR3 (Sarl, Créteil, France). This molecule is a synthetic derivative of T40 dextran in which some of the hydroxyl groups were substituted by carboxymethyl and sulfate groups (Fig. 1). The global degree of substitution (d.s.) of each polymer was determined by acid-base titration of the carboxylic and sulfate groups and by microanalysis of the sulfur content; the fine structure was determined by ^{1}H NMR spectroscopy and HPLC gel filtration-light diffusion detection (Ledoux et al., 2003). Full data were provided with the compound.

Myoblasts were supplied by Pinset and Montaras (Pinset et al., 1988). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), horse serum and 10× phosphate buffered saline (PBS)

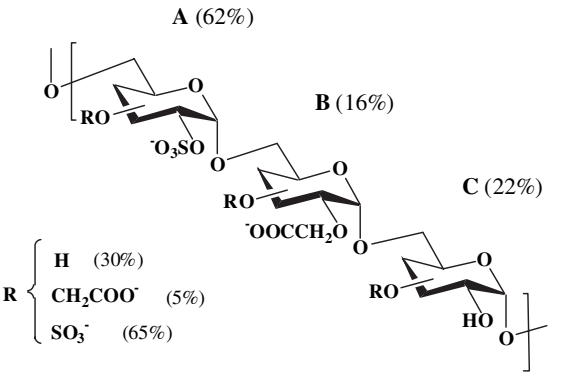

Fig. 1. Schematic structure of RGTA D120. The dextran derivative on the 1-6 glucose polymeric chain contains carboxymethyl ($–CH_2COO^{–}$) and sulfate residues ($–SO_3^{–}$) at degrees of substitution of 0.26 and 1.92, respectively. Three differently substituted glucosidic units are represented according to the nature of the group linked to the C2 position. For easier interpretation, these units were arranged in an arbitrary combination. R represents the possible substituted groups in the global C3 and C4 positions. The position of each group on the C-2 compared to C-3 + C-4 was also determined by analyzing the anomeric proton signal by ^{1}H NMR (300 MHz).

were from Gibco-BRL-Life Technologie, (Cergy-Pontoise, France). Sodium acetate, sodium formiate, formic acid, anthranilic acid and proteinase K were from Merck (Darmstadt, Germany); propan-1-ol was from Prolabo-VWR (Strasbourg, France). Ultrafree-MC Amicon filters were from Millipore (Bedford, Massachusetts). Gelatin, paraformaldehyde, p-nitrocatechol sulfate and all other chemicals were from Sigma-Aldrich (St Louis, MO). Mowiol was from Calbiochem (La Jolla, California). The protein assay kit was from Bio-Rad Laboratories (California, USA). Epitope-specific antibodies against HS were produced by the phage display technique (Jenniskens et al., 2002). Anti c-myc tag rabbit polyclonal IgG (A-14) was from Sigma, Alexa 488-conjugated goat anti-rabbit IgG was from Molecular Probes (Eugene, USA). Electrophoresis material and products were from Bio-Rad. Spectroscopic data were collected using a scanning spectrophotometer PU 8740 UV/Vis (Phillips, Paris, France). HPLC analysis was performed with a TSKgel G3000 PWXL 7.8 mm ID × 30 cm column from Tosohaas (Cambridge, UK) using an LC240 fluorescence detector from Perkin Elmer (Division Instruments, St Quentin, France); the HPLC pump was from Kontron Instruments (Germany). Analysis of HPLC peaks was performed with RadioStar software. Confocal microscopy images were acquired with a Zeiss LSM 410 laser-scanning confocal Axiovert 135M inverted microscope.

Cell cultures

The C2.7 cell line (Pinset el al., 1988) was maintained as sub-confluent monolayers in DMEM containing 1 g/l glucose and 4 mM L-glutamine supplemented with 20% FBS, 100 U/ml penicillin and 10 μg/ml streptomycin. Cells cultures were incubated at 37°C in 12% CO_2. Samples of proliferating cells (myoblasts) were taken during the following 5 days after plating. To induce differentiation, the medium was changed at sub-confluence (days 3 or 4 after plating) to DMEM supplemented with 0.25% FBS and 0.25% horse serum. Samples of differentiated cells were taken at the indicated times during the 48 hours following medium shift.

Evaluation of myoblast proliferation or differentiation

At the indicated times, cultures were trypsinized and cells were

counted in a Coulter Counter. In other cases, culture growth was determined by DNA content evaluation using a diaminophenyl indole (DAPI) assay (Brunk et al., 1979). In order to evaluate differentiation, total activity and isoform ratio of creatine kinases (CK) on cellular extracts were determined by a micro method using Biotrol reagents. Samples containing equivalent amounts of CK activity in 1 µl were loaded onto 1% agarose gels; isoforms were separated by electrophoresis and revealed by using the CK reagent (Lagord et al., 1993). Gels were illuminated with UV (365 nm) and images of the fluorescent CK bands were analyzed by the Gene Tool Syngene software. The percentages of B and M subunits were determined from these images.

Preparation of GAG extracts

At the indicated times, cellular extracts and conditioned medium were selected and treated as described previously (Barbosa et al., 2003). In brief, cellular layer extracts were performed by scraping the cells in K_2HPO_4 (50 mM, pH 8.0). Cellular extract was then digested in a solution of 50 µg/ml proteinase K in 100 mM phosphate buffer pH 8.0 at 56°C overnight. Proteinase K was then inactivated by heating the preparation for 10 minutes at 90°C. At this step, the amount of DNA in aliquot samples was determined by 4,6-diamino-2-phenylindole (DAPI) assay using salmon sperm DNA as a standard (Brunk et al., 1979). Digested tissue was then filtered through an Ultrafree-MC filter in order to eliminate interfering DNA and tissue debris from the extract. This preparation was used for sulfated GAG quantification. Conditioned medium from each plate was lyophilized and dry powder was dissolved in 100 mM phosphate buffer and treated with proteinase K as described above.

Quantification of GAG

Total sulfated GAG and HS or CS was quantified using a method based on dimethylmethylene blue (DMMB) co-precipitation with GAG (Barbosa et al., 2003). To take account of the presence of synthetic sulfated GAGs (RGTA) in extracts, the value corresponding to the added RGTA in cultures was subtracted from the total GAG measurement. HS was quantified after nitrous acid treatment of total GAG preparation, as it is known that this treatment selectively eliminates HS (Bosworth and Scott, 1994). The GAG remaining in the sample represented O-sulfated GAGs including CS. The N-sulfated GAG (HS) content was then calculated as the difference between the total GAGs and the O-sulfated GAGs in each sample.

Preparation of GAG labelled with anthranilic acid and HPLC analysis

In order to enhance sensitivity of GAG detection using HPLC, we prepared GAG samples that were tagged with anthranilic acid by reductive amination of the proteinase K-digested samples. 100 µl of proteinase K-treated GAG samples were incubated for 24 hours at 37°C under slow agitation with 200 µl of a 1:1 mixture containing 1 M anthranilic acid in ethanol and 1 M sodium cyanoborohydride ($NaCNBH_3$) prepared extemporaneously in 100 mM ammonium acetate. The reaction was stopped by addition of 300 µl of 300 mM acetate buffer at pH 4 and filtered through Ultra-free MC filters. 20 µl of the filtrate were injected in the HPLC system (TSK G3000SWXL column). GAGs were eluted at 1.0 M NaCl at 1 ml/minute and detected with an LC240 fluorescence detector. Total GAG amounts, analyzed with the Radiostar software (Berthold, Germany), represented the sum of the areas under each peak that eluted between 6 and 18 minutes. As controls, samples that were not treated with proteinase K and proteinase K alone were analyzed, applying the same procedure. In such control samples, a very weak background signal was detected in the HPLC system with respect to digested samples.

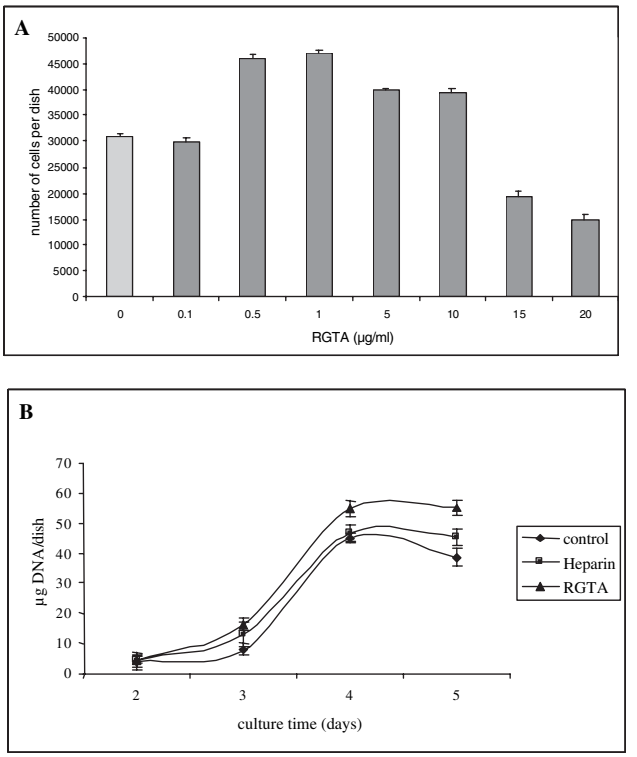

Fig. 2. Effect of RGTA on C2.7 cell growth. (A) Effect of different concentrations of RGTA (0.1 to 20 µg/ml) on cell proliferation. Cells were counted at day 4 and each value is the mean±s.d. of four cultures. (B) Growth of C2.7 myoblasts in the presence of heparin (10 µg/ml) or RGTA (0.5 µg/ml). DNA was measured in cellular extracts at the indicated times. Each point is the mean±s.d. of three determinations on three independent cultures.

Arylsulfatase assay

Determination of arylsulfatase A and B was adapted from a published method (Baum et al., 1959). In brief, cells were homogenized in 250 mM sodium acetate buffer, pH 6.0 containing 0.5% Triton X-100. The samples were kept on ice for 1 hour and centrifuged at 10,000 ***g*** for 10 minutes. Supernatants were then dialyzed for 18 hours against distilled water. Proteins were measured using BCA reagent (Bio-Rad). Arylsulfatase A was determined by colorimetry as followed: 50 µg protein from cellular extracts in water were diluted to a final volume of 50 µl in buffer A (10 mM sodium pyrophosphate, 1.7 M NaCl, 0.5 M sodium acetate, pH 5.0) and 50 µl nitrocatechol sulfate (10 mM) diluted in buffer A was added. The reaction was allowed to occur for 60 minutes at 37°C and stopped by addition of 50 µl of 1.0 M sodium hydroxide. The liberated nitrocatechol was measured at 540 nm. As a standard, 4-nitrocatechol was used between 0.250 and 5 nmol in the same buffer conditions. Aryl sulfate B was measured in similar conditions but in buffer B (0.5 M sodium acetate, 10 mM barium acetate, pH 6.0). 50 µl nitrocatechol sulfate (50 mM) in buffer B was added to the protein extract. A standard curve with 4-nitrocatechol was produced between 12.5 and 125 nmol. Two sets of assays were prepared in order to perform incubation between 30 and 90 minutes. The liberated 4-nitrocatechol was measured as described above. The evaluation of arylsulfatase B under these conditions was performed as described (Aqrabawi et al., 1993).

Real-time PCR analysis of enzymes involved in GAG metabolism

The transcription levels of most of the enzymes involved in HS

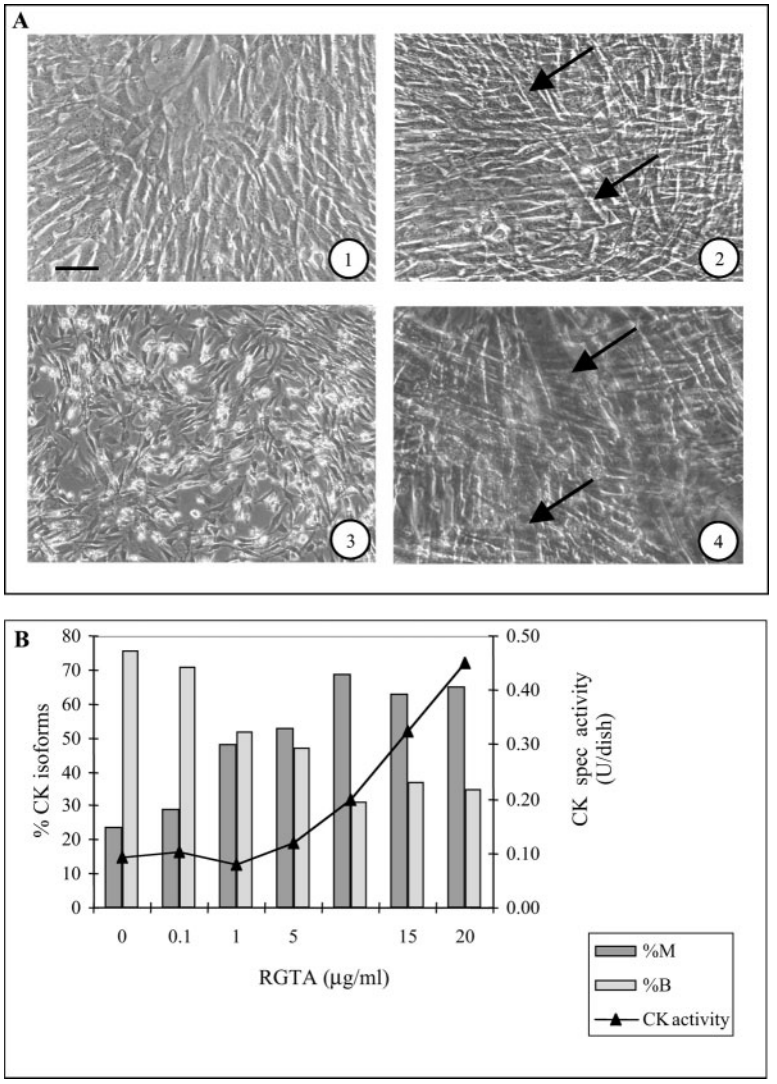

Fig. 3. Effect of RGTA on C2.7 cell differentiation. (A) C2.7 cells grown at high serum concentrations at day 4, without treatment (1) or in the presence of 0.5 µg/ml RGTA (2). C2.7 cells at 24 hours in differentiating medium (0.25% SVF + 0.25% SC) without treatment (3) or in the presence of 0.5 µg/ml RGTA (4). Arrows indicate myotubes in the cultures. (B) Subunits of creatine kinase (CK) and enzymatic activity at day 4 of C2.7 cell culture. Isoenzyme analysis was performed after agarose gel electrophoresis and the percentage of each subunit was calculated as described in Materials and Methods. CK activity is expressed as international units per dish. Bar, 100 µm.

synthesis, namely exostosin (EXT) 1 and 2, N-deacetylase N-sulfotransferase (NDST1), epimerase, 2-*O*-sulfotransferase (OST) and 6OST1, as well as heparanase involved in HS degradation, have been analyzed. Real-time PCR analyses were performed on RNA extracted from differentiating C2.7 cells at the indicated time using RNA treatment with Instapure Plus (Eurogentec, Seraingn, Belgium). The use Instapure plus permitted elimination of any interference on the RT and PCR steps which otherwise would occur owing to the presence of polyanionic molecules such as RGTA. Such a drawback has been already described for heparin (Bai et al., 2000).

The RNA pellet was dissolved in RNase-free water, treated with DNA-free (Ambion Europe, UK) and its purity verified on agarose gel. The reverse transcription reaction was performed on 1 µg total RNA from cells by using the oligo dT primer and the Superscript II^{TM} preamplification system (Invitrogen). Gene expression was quantified by real-time PCR using the LightCycler Fast Start DNA Master (Roche) with 0.2 µl cDNA, corresponding to 100 ng total RNA in a 20 µl final volume, 3 mM magnesium chloride and 0.5 µM of each primer (final concentration). Briefly, quantitative PCR was performed for 45 cycles at 95°C for 15 seconds, at the specific annealing temperature for 25 seconds and 72°C for 30 seconds. Amplification specificity was checked using a melting curve following the manufacturer's instructions. Some of the specific gene primers of each enzyme of interest for real-time PCR analysis were designed using Primers3 software based on published sequences (Table 1). Heparanase, NDST1, 2OST and 6OST1, and epimerase were used as described (Miao et al., 2002; Ford-Perriss et al., 2002; Li et al., 2002). Results were analyzed with LightCycler software v3.5 (Roche) using the second derivative maximum method to set the threshold cycle (C_T). The quantitative analysis was carried out using standard curves and normalized using the GeNorm software and methodology (Vandesompele et al., 2002). Four different reference genes [glyceraldehyde 3-phosphate dehydrogenase (GAPDH), RNA Polymerase II (RPII), TATA-box binding protein (TBP) and α-tubulin] were used.

Immunohistochemistry

The cell layers (myoblast or myotubes) were fixed with 4% paraformaldehyde for 20 minutes. Cells were washed three times with 1× PBS, treated with 2 ml ammonium chloride (50 mM) in PBS for 10 minutes and washed three times with PBS/0.2% gelatin and then incubated with anti-heparan sulfate antibodies (RB4CD12, AO4F12, AO4BO5) (Jenniskens et al., 2000) for 90 minutes. The following steps were as described previously (Jenniskens et al., 2003).

Table 1. Primer pairs for real time PCR

Gene	Accession no.	Sense	Antisense	Location	Size
NDST1	AF074926	CTTGAGCCCTCGGCAGATGC	CTACCTCACTGGGCCGTGTC	133-646	514
EXT1	BC004741	GGTCTCTCAGTCCCAGCCAGTG	AGTGGCAGGCCAGCGATGTTTG	1654-1780	127
EXT2	BC006597	GCTGTGAAGTGGGCTAGTGTGAGC	ATACTTCCACTTGTTCATCTCGTG	1485-1931	447
2OST	AF060178	ATTAAGGAGACGGAAACAAGGAG	GAAGGGTGGTGACACAGTCAAG	595-1164	570
6OST1	AB024566	ACCAGCAACTCTTTCTATCCC	AGCAATACCCACCAGCATC	2186-2720	535
Epimerase	AF003927	CCATCTATGACCTCCGGCAC	AGTCCCAGCGGGCCAG	1226-1285	59
Heparanase	NM152803	TCTGCTGCGGTGTTGAGGA	GTAATAGTGATGCCATGTAAGAGA	463-963	501
GAPDH	BU504528	ACTCCACTCACGGCAAATTC	GACACCAGTAGACTCCACGACA	201-355	155
TFIID	D01034	TACTGGAAAGGTCCCCCTCT	CTGGCTTGTGTGGGAAAGAT	1156-1367	212
RPII	BC042723	TACACCGACTCCACAAACCA	TGGACCCAATTAACGATGG	677-852	176
α-Tubulin	NM011653	CTTCCCTCTGGCCACTTATG	ACTGGATGGTACGCTTGGTC	846-1075	230

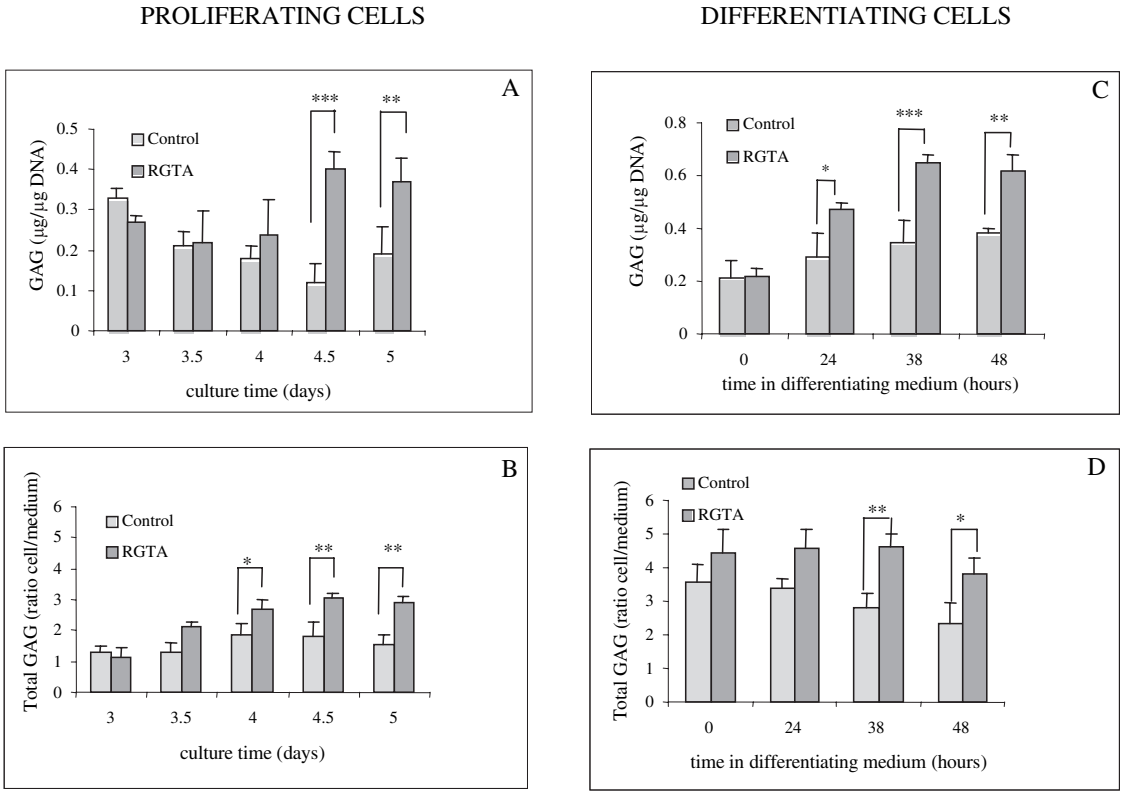

Fig. 4. Effect of RGTA on total sulfated GAG in C2.7 cellular extracts. Total sulfated GAG determinations were performed using the DMMB assay in proliferating and differentiating cells grown in the presence or absence of 0.5 µg/ml RGTA. The amounts of GAG were normalized by the amounts of DNA contained in each sample. Data are the mean±s.d. of duplicates or triplicates from four independent experiments. (A) Total GAG during proliferation. (B) Cell:medium ratio of total GAG during proliferation. (C) Total GAG during differentiation. (D) Cell:medium ratio of total GAG during differentiation. *P<0.05; **P<0.005; ***P<0.0005, when total GAG content or cell:medium ratios in RGTA-treated cells are compared with the respective controls.

Image analysis

Confocal microscopy images were acquired using a Zeiss LSM 410 laser-scanning confocal Axiovert 135M inverted microscope. For both transmission and fluorescence modes, the 488 nm emission ray of an Ar/Kr laser was used as lighting and excitation source. No emission filter was used for transmission mode but a 515 nm emission filter was used for fluorescence mode. Images of fixed cells were obtained with 63× objective with a twofold scanning zoom. Stacks of 40 slices of 0.25 µm thickness were acquired to make a maximum projection on the z-axis. The final images contain information from the base to the top of cells. Image treatment procedures (deblurring, improvement of images by unsharp mask filtering) were developed on a Macintosh computer G4 using the public domain program Object-Image, which is an extended version of NIH Image, developed at the U.S. National Institutes of Health and at the University of Amsterdam.

Evaluation of HS subspecies by ELISA

The GAG-containing extracts were used for further HS subspecies determination by ELISA. GAG was grafted on BSA as follows: 100 µl extract containing 1-10 µg/ml of GAG were mixed with 100 µl BSA (1 mg/ml) diluted in 100 mM phosphate buffer, pH 8.0 and 390 µl phosphate buffer was added. Next, 10 µl of a solution of reducing agent ($NaCNBH_3$) prepared extemporaneously at 5 mg/ml in phosphate buffer were added. The mixture was kept at 37°C overnight under moderate agitation. The BSA-GAG complex was used at 2.5 µg protein/ml to coat ELISA plates overnight at 4°C. The plates were then rinsed three times with 0.05% PBS-Tween 20. Then 3% BSA in PBS was added for 1 hour at room temperature. After three washes in PBS-Tween 20, anti HS diluted at 1:10 in 1% PBS-BSA was added. Incubation was performed at 37°C for 1.5 hours. After three washes in PBS-Tween 20, anti c-myc diluted at 1:600 in 1% PBS-BSA was added and plates were further incubated for 1 hour at room temperature. After washing, peroxidase-labeled anti IgG were added (Jackson) and plates were incubated at room temperature for 30 minutes. The plates were again washed three times and TBM kit reagent (Pierce) was used according to the manufacturer's instructions. Absorbance was measured at 450 nm. As a control, CS or HS from commercial sources were included in the assays.

Statistical analysis

Values are reported as the mean±s.d. Statistical significance was determined using one-way ANOVA non-parametric test or one-way ANOVA test followed by Dunnett's post-test using GraphPad Prism 4 (GraphPad Software, San Diego, CA).

Results

Pattern of GAG content upon C2.7 proliferation and differentiation

When C2.7 cultures were grown in serum-rich medium, cells proliferated and then spontaneously formed myotubes after 5 days of culture. Differentiation could be accelerated when serum-rich medium was replaced with low serum medium

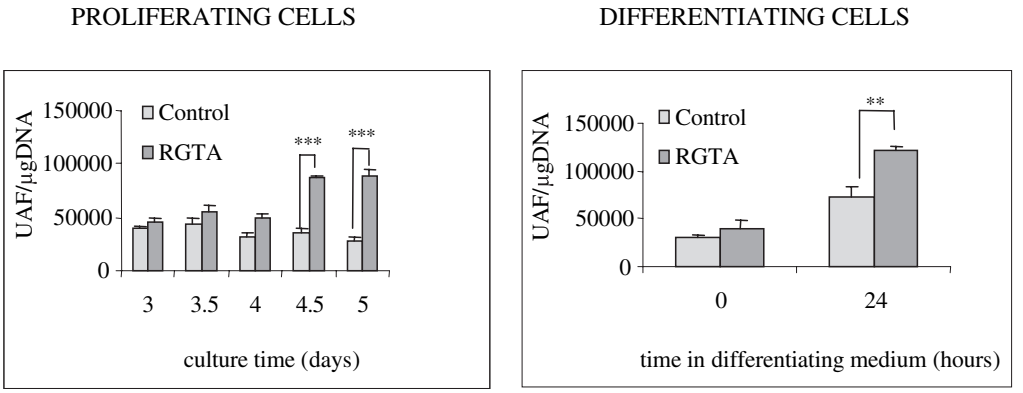

Fig. 5. Measurement of total GAG by HPLC on samples from proliferating or differentiating C2.7 cells. GAG extracts from proliferating and differentiating C2.7 cells were tagged with anthranilic acid and quantified by HPLC. Results were normalized to the amount of DNA in the samples. Data are expressed in arbitrary units of fluorescence (AUF)/µg DNA are the mean±s.d. of two independent experiments. $^{**}P<0.005$; $^{***}P<0.0005$, when compared to AUF of the respective controls.

(Figs 2 and 3). Using the DMMB technique, total sulfated GAG content was measured between days 3 and 6 in C2.7 cells grown continuously in high serum medium. We refer to cells grown under these conditions as proliferating cells. In high serum conditions, total GAG content in cellular extracts slightly decreased upon withdrawal of proliferation at days 3 and 4 after plating. A moderate increase in total GAG was noticed at day 5 upon spontaneous differentiation. This increase was further augmented twofold when cells were grown in differentiating medium (Fig. 4).

Effect of RGTA on myoblast proliferation and differentiation

Previous studies have shown that RGTA is a potent stimulating agent for proliferation and differentiation of rat satellite cells in primary cultures (Papy-Garcia et al., 2002; Stockholm et al., 1999). To provide a basis for additional studies we determined the proliferating and differentiating effects of RGTA on the C2.7 mouse cell line. As RGTA is a GAG mimetic and some of its effects were considered similar to a heparin effect, a heparin control treatment was included in most of the following experiments. RGTA applied just after plating stimulated proliferation of C2.7 myoblasts in a dose-dependent manner (Fig. 2A). The optimal doses for maximal proliferation stimulation were between 0.5 µg/ml and 1 µg/ml. The decrease in cell number for high doses of RGTA was partially due to some cell loss and to cellular differentiation, which was visible. The kinetics of cell growth in the presence of 0.5 µg/ml RGTA or 10 µg/ml heparin are shown in Fig. 2B. Compared to controls, both treatments increased cell growth, but RGTA appeared more efficient than heparin when considering product molarities. Whereas 10 µg/ml of heparin represented about 670 nM, the efficient concentration of RGTA was about 6 nM corresponding to 0.5 µg/ml. Between days 4 and 5, cell growth was stopped and cultures began to differentiate spontaneously under our culture conditions. RGTA induces precocious differentiation of cells in high serum medium (Fig. 3A1,A2). It also increased differentiation of cells that were shifted to low serum medium (Fig. 3A3,A4). Within 24 hours after medium shift from high to low serum, numerous myotubes were seen in RGTA-treated cultures (Fig. 3A4) whereas myotubes were almost absent in control cultures (Fig. 3A3). The increased differentiation in the presence of RGTA was also indicated by an increase in CK activity and an increase in the proportion of the muscular isoform (M) of this enzyme (Fig. 3B). These different results showed that RGTA increased C2.7 growth and also stimulated their differentiation. We have chosen to focus our study essentially on the effect of RGTA at the myoblast to myotube transition, i.e. between day 3 and day 6 after plating, using the RGTA concentration of 0.5 µg/ml that was effective on both processes.

RGTA increased GAG content upon proliferation and differentiation of C2.7

In proliferating cells, RGTA treatment significantly increased

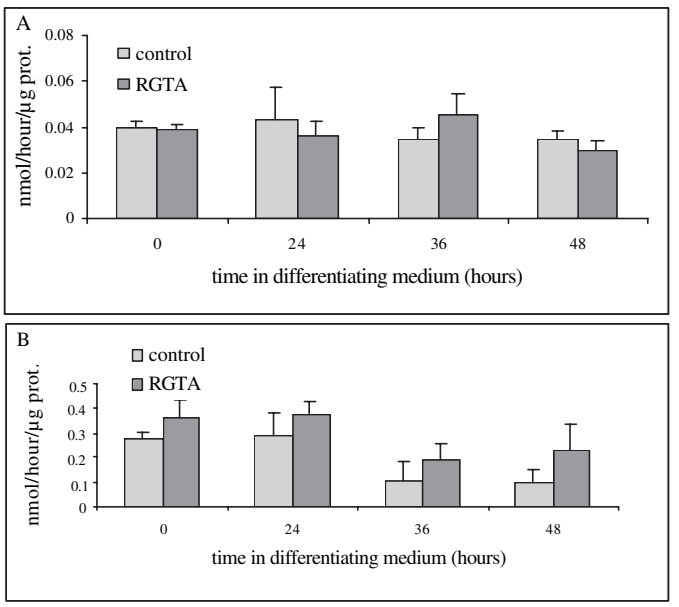

Fig. 6. Arylsulfatase specific activity in differentiating C2.7 cells. Arylsulfatase A (A) and B (B) were assayed in extracts performed at the indicated times after a shift to low serum medium. Each value is the mean±s.d. of duplicated determinations performed with two independent cultures.

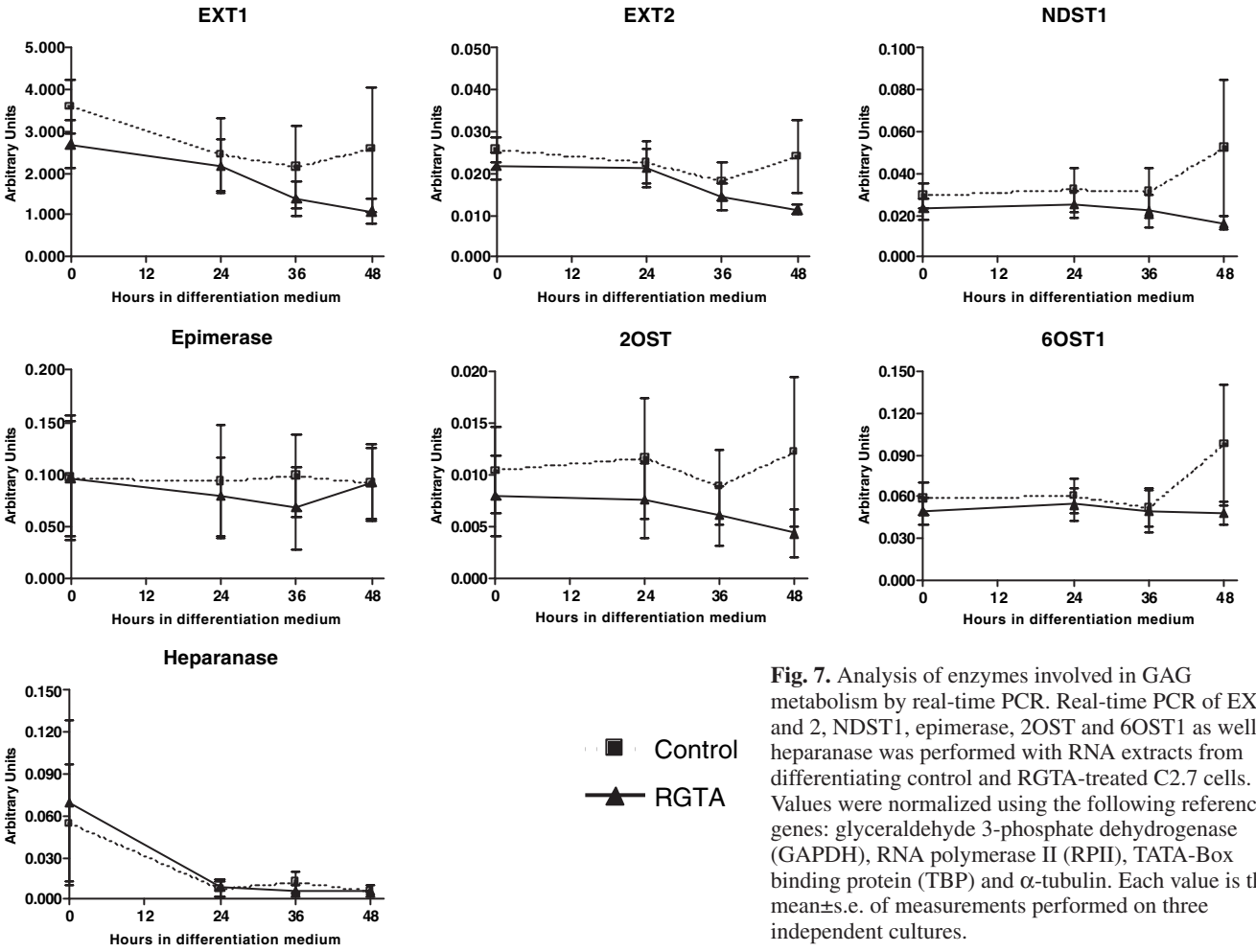

Fig. 7. Analysis of enzymes involved in GAG metabolism by real-time PCR. Real-time PCR of EXT1 and 2, NDST1, epimerase, 2OST and 6OST1 as well as heparanase was performed with RNA extracts from differentiating control and RGTA-treated C2.7 cells. Values were normalized using the following reference genes: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), RNA polymerase II (RPII), TATA-Box binding protein (TBP) and α-tubulin. Each value is the mean±s.e. of measurements performed on three independent cultures.

the amount of total sulfated GAGs between days 4 and 5 compared to controls (Fig. 4A). Whereas the cell-to-medium ratio of GAGs slightly increased from 1:1 to 2:1 under control conditions, RGTA treatment further increased the amount of GAGs in the cellular compartment compared to medium (Fig. 4B).

We also studied GAG changes in cultures in which differentiation was stimulated by a shift to a low serum medium (referred to as differentiating medium). In this medium, total GAG amount increased about twofold in controls, RGTA further increased this total GAG amount as showed in Fig. 4C. The cell-to-medium GAG ratio slightly decreased in control cultures but remained high in RGTA-treated cultures (Fig. 4D).

Sulfation rate of total GAG was not altered by RGTA

The technique used to assay total sulfated GAG content in cell extracts was based on the affinity of sulfated GAGs to the cationic DMMB dye. In order to check if RGTA increased overall GAGs, and not specifically sulfated GAGs, we performed another GAG determination using HPLC chromatography of GAG molecules tagged with a fluorescent chromophore (anthranilic acid). This method gives a signal directly proportional to the number of GAG molecules. It allowed the quantification of total GAGs, including unsulfated species such as hyaluronic acid. In these experiments, results have shown that RGTA or heparin increased the total amount of GAGs compared to controls. Heparin was always less efficient than RGTA in increasing total GAG content (not shown). This RGTA-induced increase measured in proliferating cells (Fig. 5A) or in differentiating cells (Fig. 5B) by the anthranilic acid tag technique was at similar levels to the increases measured using the DMMB assay (Fig. 4). This suggested that RGTA did not change the degree of sulfation of the GAGs. As arylsulfatase might be involved in changes of the sulfation rates of GAG, we also measured the activities of arylsulfatase A and B in control and RGTA-treated cells during differentiation (Fig. 6). Although arylsulfatase A specific activity did not significantly change in differentiating cultures (Fig. 6A), arylsulfatase B decreased in controls about fourfold over the course of differentiation. RGTA treatment of cultures did not significantly alter the activity of either sulfatase, although it seemed to slow down the decrease of arylsulfatase B in differentiating cells.

GAG metabolic enzymes during myogenic differentiation

In order to analyze the possible effect of RGTA on GAG metabolism, we determined the expression of enzymes involved in HS biosynthesis and expression of heparanase using real-time PCR in C2.7 cells under control conditions and in RGTA-treated cultures. In control cultures, the mRNA levels

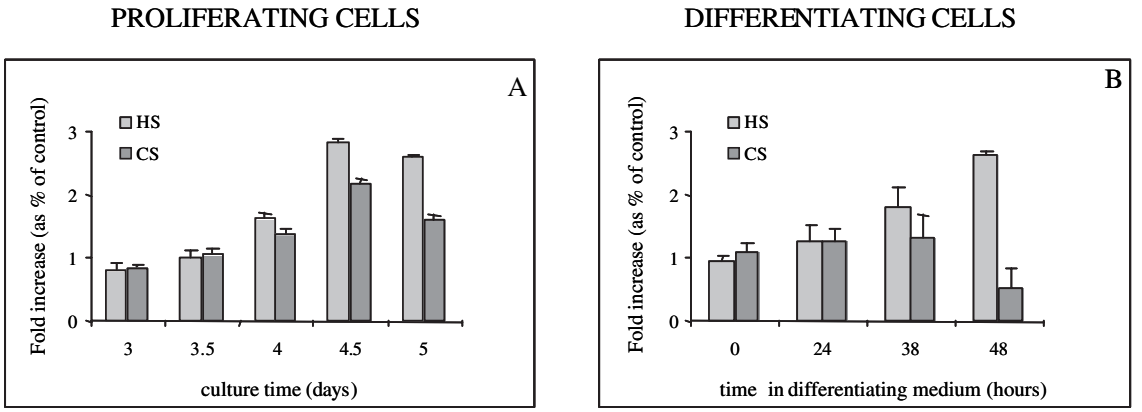

Fig. 8. Changes in GAG composition induced by RGTA in proliferating and differentiating C2.7 cells. HS and CS were measured in cellular extracts of cultures from (A) proliferating and (B) differentiating cells grown in the presence or absence of RGTA added at 0.5 μg/ml. Ratios of HS or CS contents in RGTA-treated cells compared to controls were calculated.

of most of the enzymes involved in HS synthesis, namely EXT1 and -2, NDST1, epimerase, 2OST and 6OST1 did not significantly change during the differentiation process. In the presence of RGTA, levels of the mRNAs of the enzymes involved in HS synthesis were always lower than those of controls. However, these differences were not statistically significant.

We also measured the expression of heparanase involved in HS degradation at the mRNA level (Fig. 7). Interestingly, the heparanase mRNA level decreased almost to zero in control cultures within the first 24 hours in differentiating medium. RGTA did not significantly change this pattern.

RGTA altered GAG species balance during the myogenic process

As RGTA increased the overall amounts of GAG produced by cells, we analyzed GAG composition when cells changed from the proliferating to the differentiating stages. In control cultures, CS was proportionally more important than HS during proliferation. HS increased progressively upon differentiation (not shown). In proliferating cells, RGTA increased both HS and CS contents two- to threefold when cells were post-mitotic (days 4-5; Fig. 8A). In differentiating medium, RGTA specifically increased the HS amount two- to threefold over control levels, whereas CS was not significantly altered (Fig. 8B).

HS sub-species and localization in differentiating myoblasts

It has been demonstrated that HS GAG belongs to a family of molecules whose composition in the cell environment changes during biological processes (Jenniskens et al., 2000). Using three anti-HS antibodies that recognize different HS sub-domains, we tested GAG composition with an ELISA assay (Fig. 9). The three antigens were not equally represented in cell extracts: AO4BO5 epitope seemed to be more abundant than the others. During the differentiation process, the amount of HS epitopes did not change significantly in control cultures. Interestingly, the presence of RGTA slightly modified the HS sub-species composition. Reactivity to AO4F12 antibody was

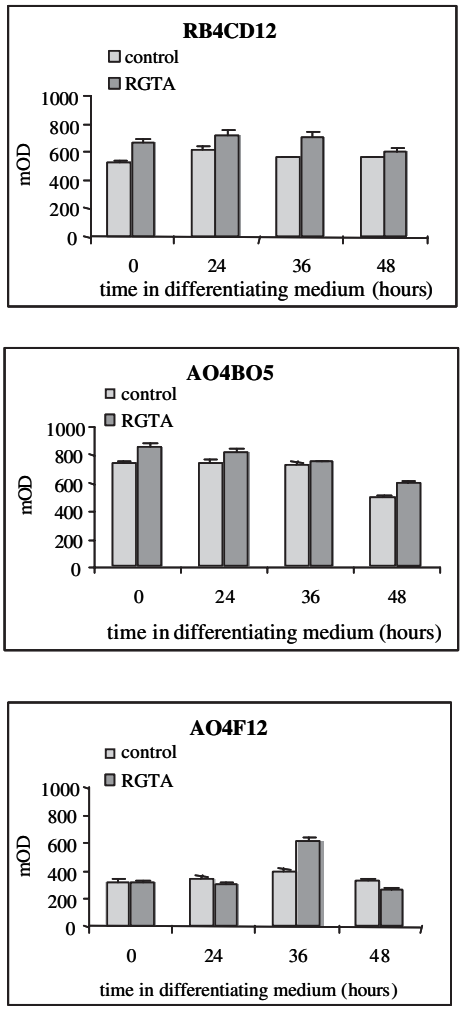

Fig. 9. ELISA assay of HS species extracted from differentiating C2.7 cells. GAG samples were prepared from differentiating controls and RGTA-treated cells of one representative culture. They were grafted on BSA and tested in an ELISA assay using different HS antibodies (RB4CD12, AO4BO5 and AO4F12). Each value is the mean of duplicate measurements.

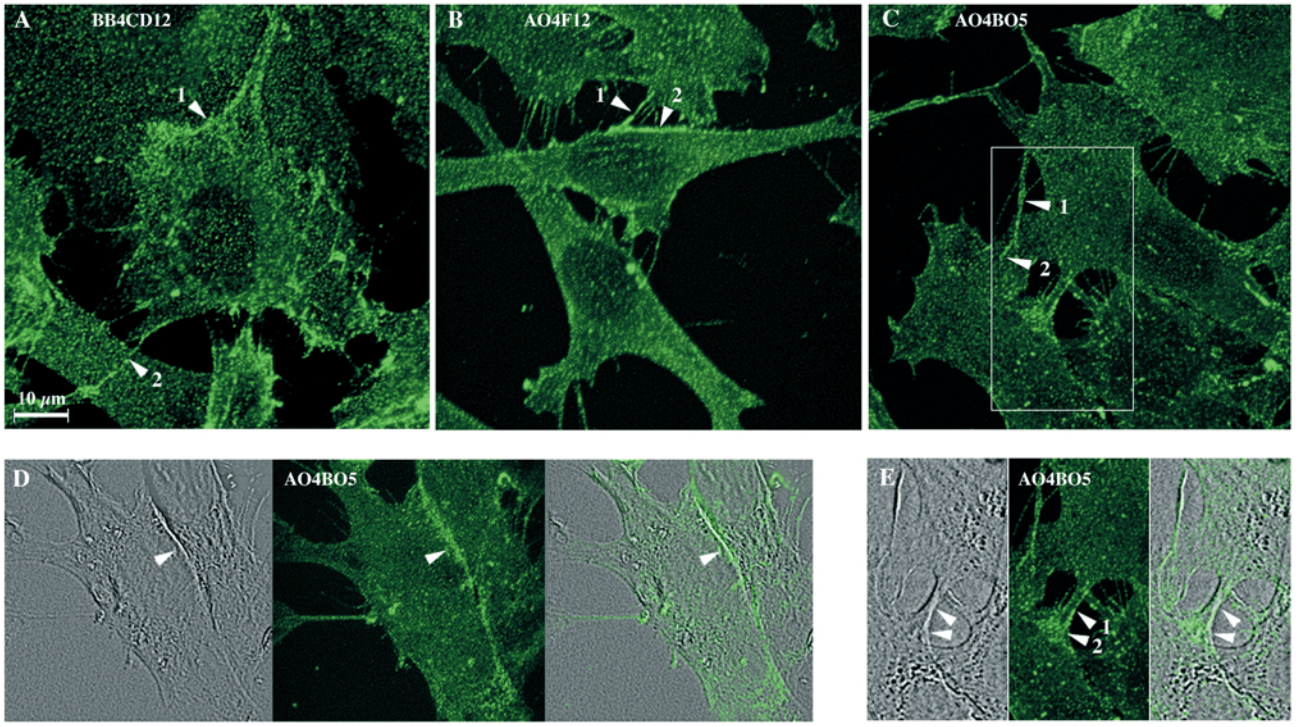

Fig. 10. Immunolocalization of HS in C2.7 myoblasts. Localization of HS subspecies was investigated using (A) RB4CD12, (B) AO4F12 and (C-E) AO4BO5 antibodies and confocal microscopy. Note the differential labelling of HS species either at the periphery of cells (RB4CD12) or at cell-to-cell contacts with AO4BO5 or AO4F12. These two antibodies labelled cell-to-cell contacts differentially. Areas indicated by arrows are described in the text. Panel E shows the boxed area in C. Bar, 10 μm.

clearly increased 36 hours after medium shift, whereas the responses to the other antibodies were only slightly enhanced compared to control levels.

Although RGTA did not seem to alter the localization of the different HS subspecies compared to controls, immunocytological analyses of these HS sub-domains revealed subtle differences in their localization in myoblasts (Fig. 10). In differentiated cultures, HS antibodies labelled a network that overlaid the cells. This network was even more accentuated in myotubes than myoblasts (not shown).

In myoblasts, the RB4CD12 antibody principally delineated a subset at the periphery of cells (Fig. 10A, arrow 1) and pseudopodia structures (arrow 2). AO4F12 (Fig. 10B) and AO4BO5 (Fig. 10C-E) antibodies decorated pseudopodia structures and cell-to-cell contacts. Pseudopodia between two cells were often labelled with AO4F12 antibody (Fig. 10B, arrow 1). Membrane close to these cell-to-cell contacts also appeared strongly labelled (Fig. 10B, arrow 2). AO4BO5 antibody also labelled pseudopodia structures (Fig. 10C, arrows 1 and 2). An intense fluorescence was also frequently observed at the contact zone between two adjacent cells (Fig. 10D, arrow). In addition, cytoplasmic bridges formed between two presumably pre-fusing cells were also labelled (Fig. 10E, arrows 1 and 2).

Discussion

It is becoming clear that the extracellular environment regulates cellular functions as complex as myogenic differentiation. GAGs are a class of molecules generally associated with core proteins to form proteoglycans and play important roles in this environment. For instance, defective heparan sulfate biosynthesis induces neonatal lethality in mice (Ringvall et al., 2000) and disturbs Ca^{2+} homeostasis in myotubes (Jenniskens et al., 2002). However, there is little available information about GAG species synthesis in the course of myogenesis. In the present study, using two methodological approaches for GAG quantification and analysis, we observed that GAG composition changed in cultures of C2.7 myoblasts when shifted from proliferation to differentiation states. These alterations concerned the overall GAG content, its distribution between medium and cell layer, and its species. We have shown that GAGs, and more especially HSs, were increased during the differentiation process. We analyzed the expression levels of most of the enzymes involved in HS metabolism. It must be emphasized that expression patterns of enzymes responsible for HS synthesis were not significantly altered during myoblast differentiation. In contrast, the level of heparanase mRNA was strongly reduced. This reduction of heparanase correlated with the increase in overall HS in differentiating cells.

The presence of RGTA, a synthetic GAG mimetic, in the cell environment increased both myoblast proliferation and differentiation. The optimal dose for enhancing proliferation was between 0.1 and 1 μg/ml. At these concentrations, a shift of B to M creatine kinase isoform, taken as a classical sign of differentiation, was observed whereas the increase in total creatine kinase activity occurred at higher RGTA concentrations. Thus, the concentration of 0.5 μg/ml mainly used here appeared the optimal dose which favoured the whole

process from proliferation to differentiation. Interestingly it was shown that RGTA emphasized the changes in GAG amount, composition and localization observed in control cultures undergoing differentiation. Heparin was always found to have a lesser effect than RGTA at equivalent concentrations.

The question of how RGTA could alter GAG synthesis and whether GAG changes are causal or the consequences of RGTA effect on proliferation and differentiation remain open. Cells are influenced by an environment composed of GAG-containing entities. The presence of RGTA, by itself, modifies this environment and thus might modify cell behavior. We believe that the changes brought by the presence of RGTA are the starting event in the process. RGTA could act first extracellularly, as do natural GAGs such as HS, by increasing bioavailability of growth factors and by potentiating growth factor-mediated proliferation and differentiation. Interestingly, we have observed, using the same RGTA labeled with FITC, that this molecule remains for several hours in the cellular environment of myoblasts as a dense network (I.M., unpublished). From this location, RGTA could mediate growth and also induce phenotypic changes in cells indirectly via growth factor modulation. We have previously shown that RGTA stimulates proliferation of satellite cells in primary cultures and that this effect was partially caused by FGF2 potentiation (Papy-Garcia et al., 2002). RGTA was also shown to partially substitute for hypo-sulfated GAG in chlorate-treated cultures and to mediate the FGF2 growth response (Papy-Garcia et al., 2002). This result could be related to the observations that chlorate-induced reduction of sulfation of HS chains of the perlecan proteoglycan in C2C12 myoblasts diminished their differentiation capacity (Villar et al., 1999). Stimulation of proliferation has been shown to correlate with an increase in matrix protein (Porcionatto et al., 1998) and HSPG secretions in endothelial cells (Weiss and Randour, 2002). Conversely, changes in HS sulfation might alter HBGF synthesis and intracellular processing. This has been demonstrated with FGF2 (Sperinde and Nugent, 1998).

The novel findings of the present study were that GAG mimetics stimulated myogenesis, along with changes in the natural GAG composition and that RGTA more particularly increased HS levels. It has long been known that TGFβ activity is regulated by proteoglycans present in the ECM, and in turn regulates synthesis of ECM components (Rapraeger, 1989; Dubaybo and Thet, 1990; Ogawa et al., 1990; Rapraeger et al., 1991; Anastassiades et al., 1996; Nishikawa et al., 2000). Similarly, growth factors, including FGF, have been shown to alter HS composition of smooth muscle cells (Emoto et al., 1998; Li et al., 2002). We hypothesize that RGTA indirectly modulates GAG patterns through FGF or TGFβ as it has been shown to protect these growth factors from proteolysis (Meddahi et al., 1996).

A possible effect of RGTA on the expression of enzymes involved in synthesis of GAG was also investigated. The mRNA of the studied enzymes did not change significantly during differentiation, and RGTA did not significantly alter the expression of these enzymes. These results suggest that changes at the transcriptional level might not be relevant for studying regulation of the enzymes involved in HS metabolism. Interestingly, the mRNA of these enzymes all have long 5′ untranslated region (5′-UTR) sequences involved in translational regulation (Grobe and Esko, 2002). This suggests a sophisticated regulation at translational levels of regulating factors that have the property of stimulating synthesis of HS and molecules that would bind to it. RGTA could interfere with this regulation.

It should be emphasized that heparanase was the only enzyme that was significantly altered in differentiating cultures whatever the treatment. We have also shown that RGTA did not modify heparanase mRNA expression. This finding does not preclude an effect at the protein level. An interesting issue that we are currently exploring is that RGTA may alter heparanase activity (A. Meddahi et al., unpublished). Such a hypothesis is supported by studies showing that heparin was able to reduce the activity of this enzyme in CHO cells (Tumova and Bame, 1997) and/or may cause a reduced intracellular degradation of HS through inhibition of endogenous heparanase as in smooth muscle cells (Potter-Perigo and Wight, 1996). RGTA was observed to enter into cells in vesicles where it remains for at least 24 hours (I.M., unpublished). We cannot rule out the fact that RGTA may further modify phenotypic cells from intracellular stores through an unknown mechanism and alter expression or activity of the enzymes involved in GAG metabolism.

The complexity of the system is further emphasized by the fact that levels of the various HS sub-species as revealed by specific antibodies were all different in RGTA and control culture conditions. We have shown that HS antibodies detect different amounts of HS sub-species in differentiating cells stimulated by RGTA. These HS antibodies, recognizing different epitopes, differentially decorated the cells. This suggests sophisticated changes in HS synthesis during myogenesis. RGTA could participate in these regulations by discretely modulating the different sets of enzymes involved in HS synthesis.

Taken together, the present results, showing effects of RGTA on myoblast growth and differentiation with alteration of GAG composition, give an important insight into the myogenesis process, especially as it occurs in a pathological situation such as skeletal muscle dystrophy or in muscle repair.

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