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Isolation and Characterization of Proteoglycans Synthesized by Rat Myoblasts L6J1 in Culture

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Abstract—Proteoglycans synthesized by rat myoblasts L6J1 in culture were isolated using sorbent Q-Sepharose from culture medium, extracellular matrix (ECM), and cells. Elution of the sorbed material in a NaCl gradient separated proteoglycans from the bulk of proteins eluted at low concentration of the salt. Four fractions (fractions I-IV) were obtained for each component of the cell culture, including two proteoglycan fractions for the ECM and culture medium and one fraction for the myoblasts. Proteoglycans of the culture medium were virtually completely represented by proteoglycans of fetal calf serum. With enzymes chondroitinase ABC and heparinase III chondroitin/dermatan sulfate proteoglycans were shown to prevail in all components of the myoblast culture. The core proteins of proteoglycans were characterized by electrophoresis.

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In a living organism, the growth and development of cells are mainly determined by their microenvironment [1, 2]. Key elements of this microenvironment are the structural and adhesive proteins, proteoglycans, growth factors, and enzymes of the extracellular matrix (ECM). Studies on interaction mechanisms of cells and ECM components are important for solution of various fundamental and practical problems associated, in particular, with mechanisms of signal transmission from the cell surface into its nucleus and the cultivation and differentiation of stem cells [3]. Proteoglycans are essential regulators of cell functions [4]. Proteoglycans are also interesting in connection with the development and regeneration of muscle tissue [5, 6]. This problem is far from being clear, although the available literature suggests a direct involvement of proteoglycans in the regulation of the growth activity of myoblasts [7-9]. Proteoglycans are a very heterogeneous group of compounds possessing tissue and cell specificity; therefore, it was important to analyze the total pool of proteoglycans produced by myoblasts.

The purpose of the present work was to isolate and characterize proteoglycans synthesized by transformed rat myoblasts L6J1 during cultivation. Special attention was given to optimization of the isolation conditions of these compounds from the myoblast culture and to general characterization of the main fractions of proteoglycans.

MATERIALS AND METHODS

Reagents. We used chondroitinase ABC, heparinase III, Alcian Blue (Sigma, USA); 1,9-dimethyl-methylene blue (DMB), phenylmethylsulfonyl fluoride (PMSF), N-ethylmaleimide (NEM) (Serva, Germany); urea (Vekton, Russia); L-[α-^{14}C]leucine (Izotop, Russia); fetal calf serum (FCS), Dulbecco modified medium (DMEM), trypsin and versene solutions (Biolot, Russia).

Abbreviations: DMB) 1,9-dimethyl-methylene blue; DMEM) Dulbecco modified medium; ECM) extracellular matrix; FCS) fetal calf serum; GAG) glycosaminoglycan(s); NEM) N-ethylmaleimide; PMSF) phenylmethylsulfonyl fluoride.

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Myoblasts and their cultivation. Transformed rat myoblasts L6J1 were obtained from the cell culture bank of the Institute of Cytology, Russian Academy of Sciences. The cells were cultured in DMEM supplemented with 10% FCS. The cells were grown in flasks (Sarstedt, Germany) with the area of 175 cm^2. The culture medium was collected during cell growth and frozen to –20°C in the presence of proteolysis inhibitors PMSF (1 mM) and NEM (10 mM) [10].

Radiolabeling. Radiolabeled amino acid L-[α-^{14}C]leucine (specific activity 1.9 GBq/mmol) was introduced into four flasks concurrently with the culture of transformed rat myoblasts L6J1 from the calculation of 1.5 MBq per flask. Upon cultivation for three days, nonradioactive and radioactive materials were combined, and proteoglycans were isolated from the cells, ECM, and culture medium by the same procedure. The radioactivity was calculated with an LS 6500 liquid scintillation counter (Beckman, Austria) at the counting efficiency of 85% using scintillation cocktail containing 100 g naphthalene, 5 g PPO, and 100 mg POPOP in 1 liter of dioxane.

Isolation of proteoglycans. Proteoglycans were isolated from the myoblast culture by a combination of methods described in [10-13]. The procedure of proteoglycan isolation from the cell culture components (the culture medium, ECM, and myoblasts) was similar and included common stages except the first stage. In the first stage, the culture medium was treated with a double volume of 50 mM Tris buffer (pH 7.0) containing 10 mM EDTA and 10 M urea. The cells and ECM were removed from the plastic using the same buffer supplemented with 50 mM EDTA, 1 M urea, and proteolysis inhibitors PMSF (1 mM) and NEM (10 mM). The cells were precipitated by centrifugation for 20 min at 4°C and 100*g*. The precipitated myoblasts were treated with 50 mM Tris buffer (pH 7.0) containing 8 M urea and 0.1% Triton X-100. The cell extract was cleared by centrifugation for 20 min at 4°C and 2000*g*. Then all components of the cell culture were treated with the sorbent Q-Sepharose for 2 h with constant stirring to extract proteoglycans. The sorbent was placed into columns, which were successively washed in 50 mM Tris buffer (pH 7.0) containing 8 M urea and 50 mM EDTA and in the same buffer supplemented with 0.2 M NaCl. Proteoglycans were eluted from the Q-Sepharose in a NaCl concentration gradient (0.2-1.5 M). The gradient fractions were combined (pooled fractions I-IV) according to the elution profile, dialyzed against water, and lyophilized. Proteoglycans were determined using the dye DMB [14].

Enzymatic treatment of proteoglycans and analysis of the glycosaminoglycan (GAG) composition. Upon the dialysis and lyophilization, the pooled fractions of proteoglycans (fractions I-IV) were dissolved in buffer (pH 7.5) optimized for enzymes chondroitinase ABC and heparinase III and containing 50 mM Tris-HCl, 100 mM NaCl, 4 mM $CaCl_2$. From each fraction, four samples were prepared 50 μl in volume containing 10 μg GAG: 1) control, without enzyme; 2) 0.4 U/ml heparinase III, 25°C; 3) 0.5 U/ml chondroitinase ABC, 37°C; 4) 0.4 U/ml heparinase III, 25°C; then 0.5 U/ml chondroitinase ABC, 37°C. GAG was enzymatically cleaved for 5 h, then 10 μl of the untreated sample and of the sample treated with chondroitinase ABC were analyzed by electrophoresis. The reaction was followed by reduction of the GAG concentration, which was determined by staining with DMB. The GAG content was determined by the difference in the optical density of the untreated sample and the sample under study by to the formula:

$$\%\,\mathrm{GAG} = \frac{A^0_{525} - A'_{525}}{A^0_{525}} \cdot 100\ \%,$$

where A^0_{525} is the optical density of the control at 525 nm and A'_{525} is the optical density of the sample upon the treatment with any of the enzymes.

Electrophoresis. Electrophoresis was performed in 7% SDS-polyacrylamide gel [15]. The samples were dissolved in 60 mM Tris-HCl (pH 6.8) supplemented with 10% glycerol and 0.5 M 2-mercaptoethanol. The gels were successively stained with Coomassie R-250 and Alcian Blue.

RESULTS

Isolation of proteoglycans by ion-exchange chromatography. Proteoglycans were isolated from 10^9 myoblasts. The gradient fractions obtained by the separation of proteoglycans on Q-Sepharose were combined according to the elution profile, and this resulted in four pooled fractions of proteoglycans from the ECM, cells, and culture medium (Fig. 1). At the NaCl concentration of 0.3 M in all cases, the protein fraction was eluted without proteoglycans. The second fraction (0.4-0.5 M NaCl) contained the major part of the protein with a small amount of carbohydrates. From the ECM and culture medium, the bulk of proteoglycans was eluted at the NaCl concentrations of 0.6 and 0.8 M as two peaks (fractions III and IV, respectively). The cells gave a single peak of proteoglycans (0.8 M NaCl), and no peak was revealed at 0.6 M NaCl.

The radioactivity curves, which allowed us to follow the proteoglycan elution by the core proteins, virtually coincided with the curves of protein contents in the ECM and cells determined by the Bradford method (Fig. 1). The radioactivity of the culture medium fractions was comparable with the background radioactivity.

To determine a possible contribution of the FCS proteoglycans to the material isolated from the culture medium, proteoglycans were isolated from the DMEM medium supplemented with 10% FCS in a volume comparable with that of the culture medium. Both the peak

height and ionic strength (0.8 M NaCl) corresponding to the major proteoglycan peak were similar to the corresponding characteristics of the culture medium (Fig. 2).

Analysis of GAG composition of proteoglycans. The GAG composition in the fractions containing the greatest amounts of proteoglycans (fourth fraction for ECM and third fraction for cells) was analyzed using enzymes chondroitinase ABC specific for chondroitin-4- and chondroitin-6-sulfates and dermatan sulfates and heparinase III specifically cleaving heparan sulfates (table). Glycosylated moiety of proteoglycans of these fractions was found to be mainly represented by chondroitin/dermatan sulfates. Thus, the enzymatic treatment of proteoglycans from the ECM and cells revealed a prevalence of chondroitin/dermatan sulfates compared to heparan sulfates—2.6 : 1 for the cells and 2.5 : 1 for the ECM (table). The culture medium proteoglycans were virtually 100% chondroitin/dermatan sulfates.

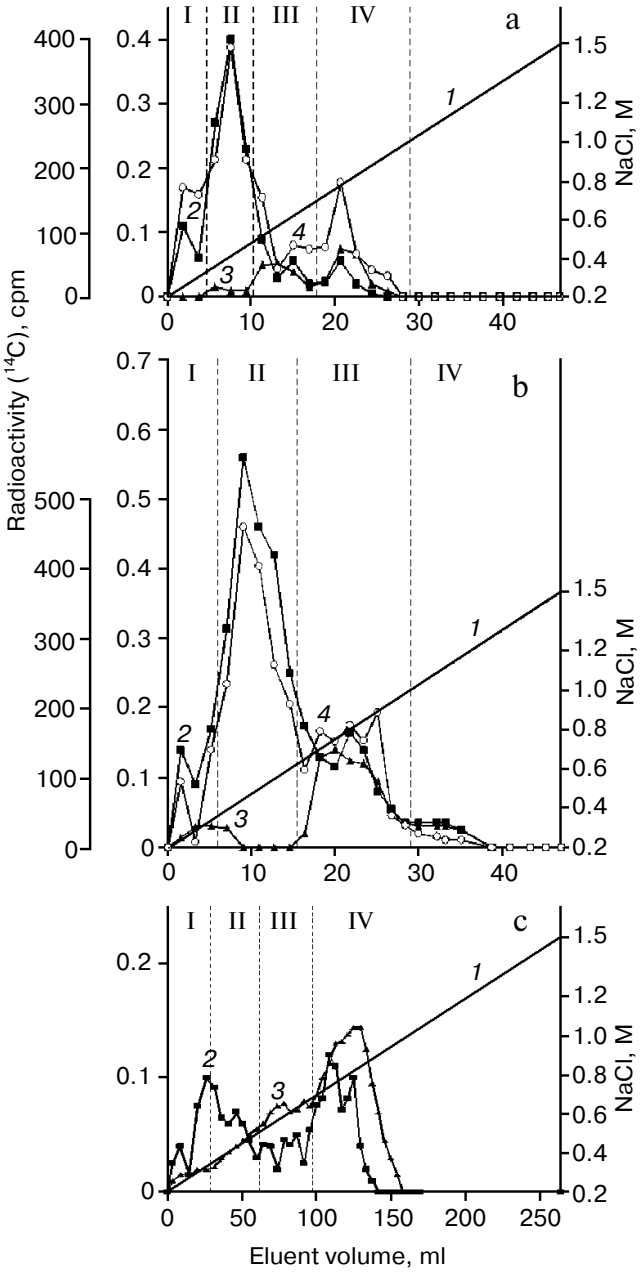

Fig. 1. Elution profiles from Q-Sepharose of proteoglycans of extracellular matrix (a), cells (b), and culture medium (c) of transformed rat myoblast L6J1 culture. Curves: *1*) NaCl concentration gradient; *2*) protein concentration in gradient fractions (A_{595}); *3*) proteoglycan concentration in gradient fractions (A_{525}); *4*) radioactivity of fractions. I-IV) pooled fractions.

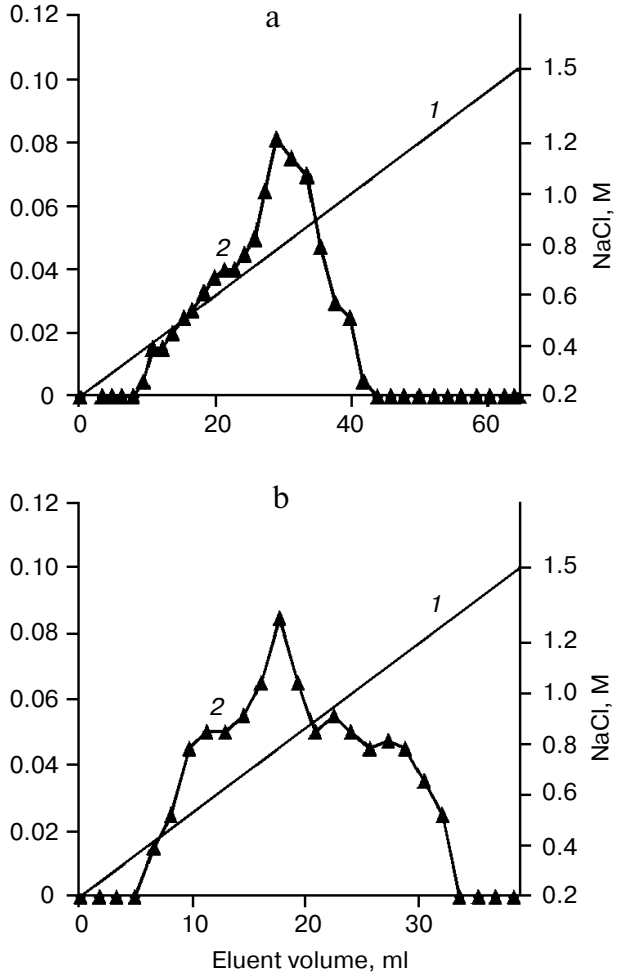

Fig. 2. Elution profiles from Q-Sepharose of proteoglycans from the culture medium of the transformed rat myoblast L6J1 culture (a) and from fetal calf serum (b). Curves: *1*) NaCl concentration gradient; *2*) proteoglycan concentration in gradient fractions (A_{525}).

Analysis of core proteins of proteoglycans. To visualize the protein and carbohydrate components, gels were successively treated with Coomassie R-250, which stained proteins violet, and Alcian Blue, which produced blue complexes with negatively charged polysaccharides. Because different dyes were used for proteoglycan staining during their isolation (DMB) and electrophoresis (Alcian Blue), it was necessary to analyze the correlation between the proteoglycan staining with these two dyes.

Carbohydrate composition of proteoglycans in major fractions of myoblast L6J1 culture

GAG content, %	Culture medium (fraction IV)		Cells (fraction III)		ECM (fraction IV)	
	heparinase III	chondroitinase ABC	heparinase III	chondroitinase ABC	heparinase III	chondroitinase ABC
Heparan sulfates	8.7	—	19.6	—	26.1	—
Chondroitin/dermatan sulfates	—	91.3	—	50.9	—	65.2
Heparan sulfates + + chondroitin/dermatan sulfates	100		70.5		91.3	

Figure 3 presents electrophoregram of proteoglycans from ECM (fractions I-IV) and myoblasts (fractions I-IV). Staining the polyacrylamide gel with Alcian Blue almost fully repeated the staining with DMB of the proteoglycan fractions in the elution curves (Fig. 1). Thus, proteoglycans of fraction III of cells and those of fractions III and IV of ECM reacting with DMB during the isolation were stained with Alcian Blue in the gel. The only exception was fraction II of the myoblast proteoglycans, which was stained blue in polyacrylamide gel, but in solution this fraction was not stained with DMB.

Core proteins of proteoglycans were revealed by comparing the electrophoregrams of the proteoglycan fractions before and after treatment with enzymes. The culture medium proteoglycans were suitable for elaboration of the procedure of sample treatment with the enzymes (Fig. 4). Fraction IV of the culture medium containing the maximum amount of proteoglycans was treated with heparinase III and chondroitinase ABC. Sample treatment with heparinase III specific to heparan sulfates, which were absent in the culture medium proteoglycans (table), did not change the picture obtained for the control sample not treated with the enzyme. On the contrary, upon the treatment with chondroitinase ABC, the blue staining of the lane in polyacrylamide gel disappeared, but two protein lanes appeared with molecular weights of 90 and 45 kD. Moreover, upon treatment with chondroitinase ABC of the culture medium fraction IV, new protein lanes appeared in the region of molecular weights from 50 to 75 kD (Fig. 4, lane *2*). The electrophoretic control of the chondroitinase ABC preparation (Fig. 4,

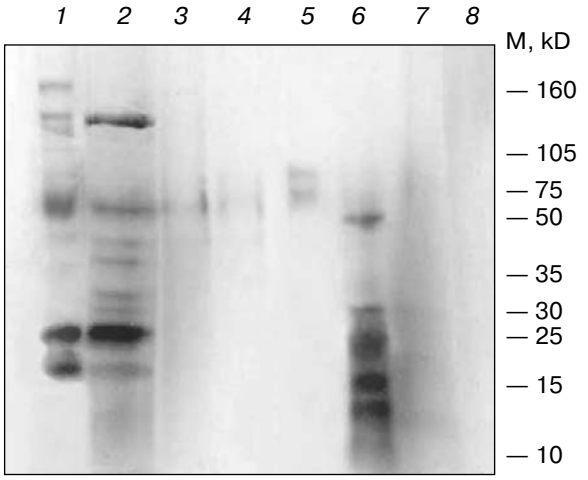

Fig. 3. Electrophoresis in 7% SDS-polyacrylamide gel of proteoglycan fractions of ECM and myoblasts resulting upon the separation on Q-Sepharose. Lanes: *1-4*) fractions of myoblast proteoglycans; *5-8*) fractions of ECM proteoglycans.

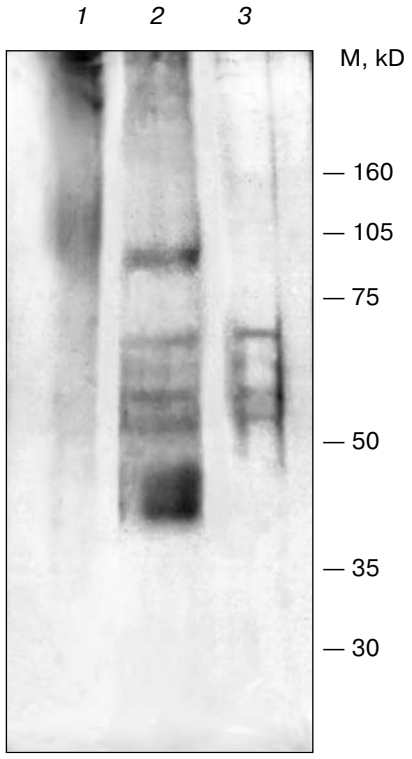

Fig. 4. Electrophoresis in 7% SDS-polyacrylamide gel of fraction IV of culture medium proteoglycans after treatment with chondroitinase ABC. Lanes: *1*) control, without enzyme; *2*) treatment with chondroitinase ABC; *3*) chondroitinase ABC (control).

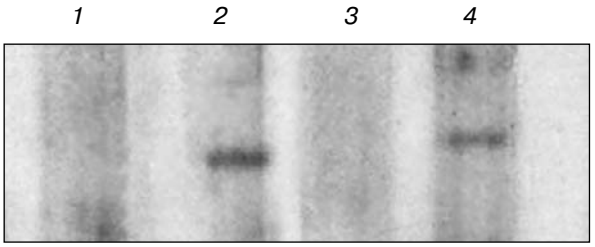

Fig. 5. Electrophoresis in 7% SDS-polyacrylamide gel of the main proteoglycan fractions from myoblasts (fraction III) and ECM (fraction IV) after treatment with chondroitinase ABC. Lanes: *1*) fraction III of myoblasts (control, without enzyme); *2*) fraction III of myoblasts treated with chondroitinase ABC; *3*) fraction IV of ECM (control, without enzyme); *4*) fraction IV of ECM treated with chondroitinase ABC.

lane *3*) revealed the same lanes, which could be explained by the presence of BSA added in the concentration of 0.01% on dissolving of the lyophilized enzyme preparation to stabilize it, as recommended by the producer.

The data presented in the table indicate that the major proteoglycan fractions of ECM (fraction IV) and myoblasts (fraction III) mainly consist of chondroitin/dermatan sulfates. The treatment of these fractions with chondroitinase ABC, which cleaved proteoglycans of both classes, allowed us to reveal the core proteins of proteoglycans by electrophoresis. The molecular weights of these proteins were insignificantly different, being 63 kD (ECM fraction IV) and 55 kD (myoblast fraction III) (Fig. 5). The treatment of these fractions with heparinase III, as in the case of the culture medium proteoglycans, failed to change the electrophoretic pattern compared to the control. Treatment with the mixture of chondroitinase ABC and heparinase III of the "anomalous" fraction II of the myoblast proteoglycans stainable with Alcian Blue in polyacrylamide gel and not stainable with DMB during the gradient analysis gave no differences compared to the control sample.

DISCUSSION

Studies on the role of proteoglycans in vital activity of cells are associated with the necessity to isolate these compounds from different tissue compartments. Cell cultures are a good model for such investigations. The literature suggests considerable differences in the composition and functions of proteoglycans of the culture medium, cells, and ECM [10, 13, 16, 17]. Therefore, it is necessary to study separately proteoglycans of culture medium, cells, and ECM. The use of Q-Sepharose for isolation of proteoglycans is an effective approach for proteoglycan sorption from biological material and their subsequent fractionation in a NaCl concentration gradient [10, 13, 18, 19]. The use of specific radiolabeled compounds introduced into the culture medium during the cultivation of cells for labeling the core proteins or polysaccharide chains of proteoglycans facilitates detecting proteoglycans during isolation [13, 17, 20]. Specific dyes staining GAG, such as DMB and Alcian Blue, are used alternatively [13, 14, 21]. We have combined the radiolabeling with ^{14}C-leucine of the proteins synthesized by the cells and the specific dyes for detecting GAG: DMB during protein isolation and Alcian Blue during electrophoresis.

The use of Q-Sepharose allowed us to isolate the majority of proteins from proteoglycans and partially separate proteoglycans themselves (Fig. 1). Comparison of the elution profiles from Q-Sepharose revealed both common features and differences in proteoglycans from the components of the myoblast culture. The common features were obvious on analyzing the curves of the GAG contents in proteoglycans, whereas the differences became clear on comparing the curves describing the GAG contents in proteoglycans. The common features of the elution profiles during proteoglycan isolation from different components of the cell culture were represented by two protein peaks eluted at low NaCl concentration (0.3 and 0.4-0.5 M). These peaks were virtually not overlapped with the proteoglycan peaks, and this allowed us to separate the protein fraction from those of proteoglycans. The major peak of proteoglycans of the culture medium, ECM, and myoblasts was also eluted at approximately the same concentration of NaCl (0.8-0.9 M). This fact seemed to be due to the belonging of this fraction of proteoglycans to the same class, which was prevalent in all components of the myoblast culture, although the bulk of the culture medium proteoglycans was represented by proteoglycans of FCS. As differentiated from the myoblast proteoglycans, which gave only one peak on fractionation, the elution profile in the case of ECM had two peaks of proteoglycans. The curves characterizing the radioactivity of the gradient fractions virtually coincided with the curves describing the protein contents in the fractions (A_{595}). For proteoglycans (A_{525}), there was a coincidence with the radioactivity profile in the case of the myoblasts (Fig. 1b), whereas in the case of the ECM there was not complete coincidence (Fig. 1a). These data suggest that the rate of metabolism of the core proteins of individual proteoglycan fractions from ECM could be different.

The presented ion-exchange chromatograms of the material sorbed on Q-Sepharose obtained for the components of the culture of transformed rat myoblasts L6J1 were rather like the pattern described by us for the primary culture of embryonal myoblasts of rat skeletal muscles [22, 23]. Thus, proteoglycans of ECM of embryonal myoblasts were also eluted at NaCl concentrations in the range of 0.6-0.9 M. Proteoglycans isolated from the embryonal and transformed myoblasts L6J1 were eluted at the same concentration of NaCl (0.8 M). The protein fractions of the ECM and cells in both cases were eluted

as two peaks at low concentrations of NaCl (0.25 and 0.4-0.5 M).

The elution profiles obtained by ion-exchange chromatography of proteoglycans isolated from other cell types have much in common with our data [17, 18, 24]. The authors revealed one or two protein peaks eluted at low concentrations of NaCl: 0.2-0.25 and 0.4-0.5 M. As a rule, proteoglycans are eluted as two peaks, the first of which is represented by heparan sulfate proteoglycans and the second peak is formed by chondroitin/dermatan sulfate proteoglycans [17-19]. Proteoglycans were eluted at NaCl concentrations in the range 0.5-1.0 M. The pattern of proteoglycan elution depends on cell type, ion-exchange sorbent, and eluent.

The presence of chondroitin sulfate proteoglycans in human serum [25] led us to perform a special study related with the proteoglycan composition of the culture medium. Such proteoglycans were found to prevail in the FCS used by us: the treatment with chondroitinase ABC almost completely cleaved GAG of serum proteoglycans (table). Results of the treatment of the gradient fractions with heparinase III and chondroitinase ABC showed that the major proteoglycan fraction of ECM, cells, and culture medium mainly consist of chondroitin and/or dermatan sulfate proteoglycans (table). These data are in agreement with data on proteoglycans of chicken primary myoblast culture [26]. Up to 90% of proteoglycans of these myoblasts consists of chondroitin sulfate proteoglycans.

Some GAG of ECM and cells were not cleaved by the enzymes as indicated by retention of the staining with DMB (table). This could depend on the presence in the proteoglycans under study of keratan sulfates not degradable by heparinase III and chondroitinase ABC [13, 27]. Results of electrophoretic analysis of the proteoglycan fractions were consistent with the elution curves from Q-Sepharose except fraction II of the cells, which according to the elution curve (Fig. 1b) contained only the protein component, was stained in the gel by Alcian Blue (Fig. 3, lane *2*). This discrepancy seemed to be caused by the presence in cell fraction II of hyaluronic acid lacking sulfo groups that prevented the interaction of hyaluronic acid with DMB [14]. Alcian Blue has no such a specificity for sulfo groups as DMB and can stain polyanions not containing sulfo groups, in particular, hyaluronic acid [13].

Alcian Blue used for staining proteoglycans (Fig. 3) stained lanes light blue, which was specific for glycosylated proteins [13]. Enzymatic cleavage of the carbohydrate moiety of proteoglycans released the core proteins of proteoglycans: the light blue staining of the lanes disappeared and separate bands appeared. After enzymatic treatment of culture medium proteoglycans, two new bands appeared with molecular weights of 90 and 45 kD, which were supposed to be core proteins of FCS proteoglycans. The new protein bands of culture medium fraction IV in the range 50-75 kD (Fig. 4, lane *2*) were shown to depend on the presence of BSA in the preparation of chondroitinase ABC (Fig. 4, lane *3*), which was added for the enzyme stabilization on its dissolution, in accordance with the producer's recommendations. The treatment with chondroitinase ABC of fraction IV of ECM and fraction III of myoblasts containing the maximal amounts of proteoglycans eluted at the same ionic strength (0.85 M NaCl) revealed 63- and 55-kD core proteins, respectively (Fig. 5, lanes *2* and *4*).

Thus, in the present work proteoglycans have been isolated and fractionated from different components (culture medium, cells, and ECM) of transformed rat myoblasts L6J1 in culture. The core proteins and carbohydrate moieties of the isolated proteoglycans have been characterized. Further work in this line will allow us to characterize individual proteoglycans synthesized by myoblasts in culture and study the involvement of these compounds in proliferation and differentiation of myoblasts.

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