Changes in the Sulfation Extent of Membrane-Associated Proteoglycans Produced by Sertoli Cells in Culture

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Abstract   Sertoli cells in culture synthesize two different membrane-associated proteoglycans (MA-PG): a proteoglycan containing heparan sulfate (HS) and chondroitin sulfate (CS) glycosaminoglycan (GAG) chains and a CS-PG containing only CS-GAG chains. The structure of these molecules is regulated by the presence of fetal calf serum (FCS) in the culture medium. Changes in the concentration of FCS resulted in changes in the total $^{35}SO_4$ incorporation into MA-PG and a shift in the elution profile of each component subjected to ion-exchange chromatography. Thus, without FCS, the incorporation was low, while in 1% and 10% FCS, the uptake of the precursor was 1.7 and 4.5 times higher, respectively. MA-PG synthesized by Sertoli cells cultured in 10% FCS eluted from DEAE-Sephacel columns at higher salt concentration than the MA-PG synthesized by cells cultured in 0% or 1% FCS. Double-labeled experiments showed that the $^{35}SO_4/^3H$-glucosamine ratio incorporated into MA-PG produced by Sertoli cells, increased from 17.6 to 23.6 and 50.9 in cells cultured at 0, 1, and 10% FCS, respectively. However, the presence of FCS affected neither the hydrodynamic size nor the chemical nature of GAG chains of MA-PG. These results show that changes in the FCS concentration promote changes in the sulfation extent of MA-PG molecules produced by Sertoli cells.   © 1995 Wiley-Liss, Inc.

Key words: proteoglycans, sulfation, serum, Sertoli cells, fetal calf serum

Sertoli cells in culture synthesize different extracellular matrix components (ECM), such as fibronectin, laminin, collagen [Rodríguez et al., 1991; Skinner et al., 1985], and two proteoglycans (PGs): a PG containing CS and HS chains and a PG containing only CS chains [Rodríguez and Minguell, 1989; Skinner and Fritz, 1985]. PGs produced by Sertoli cells are located both intra- and extracellularly, as well as associated to the cell membrane (MA-PG) [Rodríguez and Minguell, 1989]. Previously, we reported that MA-PG are not randomly distributed on the membrane surface. In fact, we observed a polarized distribution pattern [Rodríguez and Minguell, 1992a]. This distribution suggests that MA-PGs play specific roles in the interactions of Sertoli cells with the cellular or the ECM environment.

The different distribution may also implies a differentially regulated synthesis for each PG. Thus, in addition to age-related changes in the

amount of the MA-PG, we also reported changes of MA-PG synthesis after interaction of Sertoli cells with collagen substrate [Rodríguez and Minguell, 1989, 1992b].

In other somatic cells subjected to different treatment in culture, it has also been shown changes in the structure of the PG molecules. The latter has been reported to occur in several cell systems, where the action of various regulatory molecules, or the proliferation status of the cells, induce changes in the sulfation of PG [David and Van Den Berghe, 1983; Hutchison and Yasin, 1986; Robinson et al., 1984; Preston et al., 1985; Fedarko and Conrad, 1986], addition of glycosaminoglycans (GAG) chains to the core protein [Nurcombe et al., 1993; Rapraeger, 1989], and increase of GAG chains length [Nurcombe et al., 1993].

In Sertoli cells, there is no information related to possible changes in the structure of these molecules as a response to regulatory molecules or in cells subjected to different culture conditions. Thus, this work studied whether changes in the culture conditions were able to produce changes in the structure of MA-PG produced by Sertoli cells. For these purposes, Sertoli cells

were cultured at different fetal calf serum (FCS) concentrations, generally employed in cell cultures as a source of different hormones and growth factors.

Results show that the MA-PG synthesized by Sertoli cells in culture change their sulfation extent. These changes are dependent on the concentration of FCS present in the culture medium. However, no difference were observed in the hydrodynamic size nor in the chemical nature of the GAG moieties of MA-PG synthesized by Sertoli cells under the different culture conditions used here.

MATERIALS AND METHODS
Preparation and Culture Conditions of Sertoli Cells

Sertoli cells from Wistar pubertal (40–45-day-old) rats were used in these experiments. Cells were isolated as previously described [Hutson and Stocco, 1981; Rodríguez and Minguell, 1992a]. Briefly, the testes were decapsulated and treated with a collagenase solution, 0.4 mg collagenase/ml in Medium 199 containing 0.1% bovine serum albumin (BSA), type I Worthington, for 30 min at 37°C. After this enzymatic treatment, the seminiferous tubules were manually isolated and treated again with the collagenase solution for an additional 30 min at 37°C. Sertoli cells were mechanically dispersed by repeated pipetting and resuspended in Medium 199 supplemented with 12% FCS, 12% horse serum, 0.1 μM hydrocortisone, 100 U/ml penicillin, and 100 μg/ml streptomycin (culture medium). Cells (6×10^5 cells) were seeded into culture dishes (60 mm, Nunc) and incubated at 33°C in a humidified atmosphere of 5% CO_2. The culture medium was replaced by fresh culture medium every 3 days. Under these conditions, aggregates with morphological characteristics of Sertoli cells were evident in the adherent layer after day 3 in culture. Peritubular myoid-like cells and fibroblasts represented only 5% of cells in the adherent layer [Rodríguez and Minguell, 1992a].

Metabolic Labeling of MA-PG

After 12 days in culture, Sertoli cells were used for PG synthesis studies. For these purposes, cells were incubated in culture medium supplemented with different FCS concentrations (0%, 1%, and 10%) and with 5 μCi/ml ^{3}H-glucosamine (ICN Biomedical Inc., Irvine, CA;

40 Ci/mmol) and/or 20 μCi/ml sodium ^{35}S-sulfate (ICN, 43 Ci/mg S) as GAG radioactive precursors. At the end of the labeling period (48 hr at 33°C), the culture medium was removed, and Sertoli cells in the adherent layer were washed twice with phosphate-buffered saline (PBS). The radioactive material present in the culture medium as well as in the washes was discarded.

Preparation and Characterization of MA-PG

MA-PG were obtained as previously described [Oohira and Wight, 1983]. Briefly, Sertoli cells were exposed to a mild treatment with trypsin (0.05%, 10 min at 37°C). The released cells were collected by centrifugation and the supernatant, referred to as the membrane-associated (MA) fraction, containing the MA-PG was saved. The cells were washed two times with PBS and the washes were pooled with the MA fraction. To the MA fraction, 0.4 g/ml of solid guanidine–HCl, 1.25 mg/ml of N-ethylmaleimide, and 0.18 mg/ml phenylmethylsulfonylfluoride were added [Oohira et al., 1983].

To remove the unincorporated radioisotopes, guanidine–HCl, and other chemicals, the MA fraction was applied into a Sephadex G-50 column (0.8×15 cm) equilibrated with a buffer containing 8 M urea, 0.05 M sodium acetate, 0.15 M sodium chloride, and 0.5% Triton X-100, at pH 7.0 (buffer A).

Ion-Exchange Chromatography

To analyze the charge density, and therefore the sulfation extent of MA-PG, fractions containing the excluded material (Vo) from Sephadex G-50 were pooled and loaded into a DEAE–Sephacel column (0.6×10 cm) equilibrated and washed with buffer A. The retained material was eluted with a linear gradient of sodium chloride (0.15–0.8 M) prepared in buffer A. Fractions (0.8 ml) were collected and aliquots taken for measurements of radioactivity and sodium chloride content.

Molecular Sieve Chromatography

To analyze the hydrodynamic size of MA-PG, the species eluted from the DEAE–Sephacel column were pooled, dialyzed exhaustively against distilled water, and finally concentrated by lyophilization to dryness. The lyophilized material was reconstituted in buffer A and subjected to rechromatography on DEAE–Sephacel. The spe-

cies eluted from the latter DEAE–Sephacel col-
umn were dialyzed against distilled water; ly-
ophilized to dryness; reconstituted in a solution
containing 4 M guanidine–HCl, 0.05 M sodium
acetate, 0.5% Triton X-100, at pH 6.0 (buffer B);
and applied to a Sepharose CL-6B column
(0.5 × 100 cm) [Chang et al., 1983]. After elu-
tion with buffer B, fractions (0.45 ml) were
collected and aliquots taken for radioactivity
measurements.

Glycosaminoglycan Chain Characterization

The fractions containing the PG molecules
eluted from the Sepharose CL-6B columns were
pooled, dialyzed exhaustively against water, and
lyophilized to dryness. The lyophilized material
was reconstituted in water and treated with 0.1
M sodium hydroxide in the presence of sodium
borohydride (75.5 mg/ml), to remove the GAG
chains from the core proteins [Carlson, 1968].
The samples were incubated at 45°C for 48 hr;
after this time, the pH was adjusted to slightly
<7 with glacial acetic acid. The GAG chains
released by the alkaline borohydride treatment
were analyzed by Sepharose CL-6B chromatog-
raphy with buffer B as eluent. To further charac-
terize the GAG moieties, the released chains
were subjected to chondroitinase ABC, chon-
droitinase AC II, and nitrous acid treatments

[Shively and Conrad, 1976]. The extent of the
enzymatic and chemical reactions was deter-
mined by the appearance of low-molecular-
weight-labeled species after Sephadex G-25 chro-
matography.

RESULTS

Characterization of MA-PG Synthesized by Sertoli Cells

Ion-exchange chromatography. After la-
beling of Sertoli cells with 20 μCi/ml $^{35}SO_4$, the
MA fraction was prepared as described under
Materials and Methods. The analysis of PG in
the MA fraction by anion-exchange chromatog-
raphy revealed the presence of two different
components, which were eluted from the DEAE–
Sephacel column at 0.35 M (peak I) and 0.45 M
NaCl (peak II), respectively (Fig. 1). Sixty-five
percent of the radioactivity associated with peak
I was sensitive to chondroitinase ABC and AC II
treatments, while 35% was sensitive to nitrous
acid treatment. On the other hand, the PG elut-
ing as peak II was completely sensitive to chon-
droitinase ABC and AC II treatments and insen-
sitive to nitrous acid treatment.

Molecular sieve chromatography. The
hydrodynamic size of the MA-PG species, as
isolated by ion-exchange chromatography, was
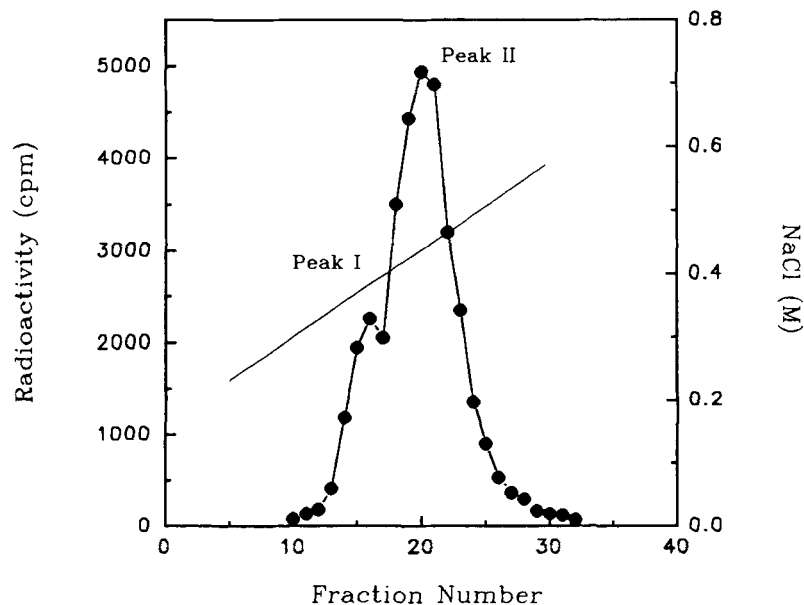studied by molecular sieve chromatography on

Fraction Number

Fig. 1. DEAE–Sephacel chromatography of MA-PG produced
by Sertoli cells cultured in 10% FCS. MA-PG were prepared by a
mild trypsin treatment, separated from unincorporated radioiso-
tope and other chemicals by Sephadex G-50 chromatography,
and the fractions containing the excluded material (Vo) were
loaded into the DEAE–Sephacel column. Retained material was
eluted with a linear gradient of sodium chloride (0.15–0.8 M).
Aliquots of column fractions were used for radioactivity and salt
content measurements.

Sepharose CL-6B columns. The radioactive material eluting from DEAE–Sephacel columns as peak I, eluted from the gel filtration column as a single peak, with a K_{av} of 0.48 (Fig. 2A). The fact that this species, after treatment with nitrous acid, eluted from a Sepharose CL-6B column with a higher K_{av} than that of the nontreated species, and the sensitivity of this material to enzymatic and chemical treatments (data not shown) strongly suggested that this species contained both CS and HS GAG chains.

The radioactive material eluted from the DEAE–Sephacel column as peak II, eluted from Sepharose CL-6B columns (Fig. 2B), as a single sharp peak with a K_{av} of 0.35, which was completely sensitive to chondroitinase ABC and AC II treatment.
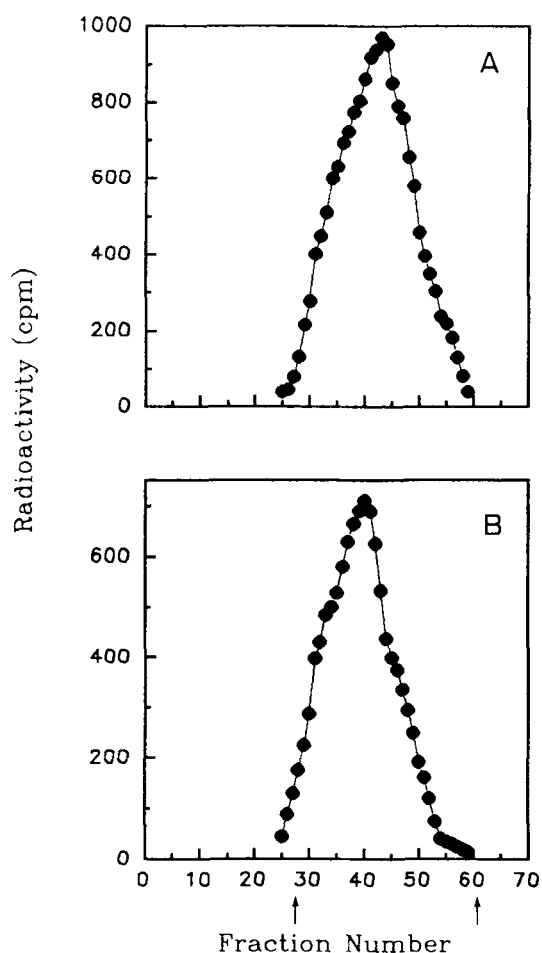
GAG chain analysis. MA-PG eluted from Sepharose CL-6B columns (Fig. 2A,B) was subjected to alkaline hydrolysis, to release the associated GAG chains. After this treatment, the radioactive materials were analyzed by Sepharose CL-6B chromatography. As shown in Figure 3A, the GAG chains derived from the component with a K_{av} of 0.48, coeluted from Sepharose CL-6B columns as a single peak with a K_{av} of 0.55, suggesting that the sizes of both types of chain, HS and CS, are very similar.

The chains released from the component eluted from Sepharose CL-6B at K_{av} of 0.35, after alkaline hydrolysis was resolved, as shown in Figure 3B, as a single peak with a K_{av} of 0.45.

Fig. 2. Sepharose CL-6B chromatography of material eluted from DEAE–Sephacel chromatography as peak I (A) and peak II (B). DEAE–Sephacel fractions pooled, dialyzed, and lyophilized were reconstituted in 4 M guanidine hydrochloride, 0.5% Triton X-100, 0.05 M sodium acetate, pH 6.0, and applied to Sepharose CL-6B column. Aliquots of the column fractions were taken for radioactivity measurements. Arrows indicate Vo and Vt.
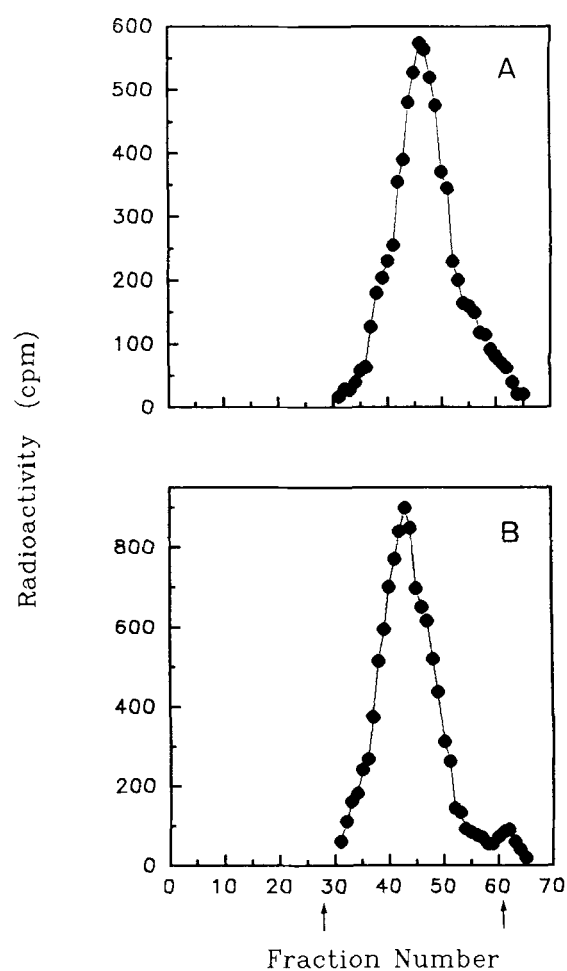
Fig. 3. Sepharose CL-6B chromatography of GAG chains liberated by alkaline borohydride treatment of material and eluted from DEAE–Sephacel as peak I (A) and peak II (B). After dyalisis and lyophilization of appropiately pooled DEAE-Sephacel fractions, the samples were treated with alkaline sodium borohydride, neutralized with glacial acetic acid and chromatographed on a Sepharose CL-6B column. Aliquots of the column fractions were taken for radioactivity measurements. Arrows indicate Vo and Vt.

Taken together, these data show that Sertoli cells in culture synthesized two types of MA-PG: (1) mixed PG (m-PG), containing HS and CS GAG chains, which eluted from DEAE–Sephacel columns as peak I and from Sepharose CL-6B with a K_{av} of 0.48; and (2) a proteoglycan containing only CS GAG chains (CS-PG), which eluted as peak II from DEAE–Sephacel columns and eluted from Sepharose CL-6B columns at K_{av} = 0.35. Table I summarizes the characteristics of MA-PG synthesized by Sertoli cells is given in.

Effect of FCS on MA-PG Produced by Sertoli Cells

Analysis of the extent of $^{35}SO_4$ incorporated *into* PGs produced by Sertoli cells shows that the incorporation of the precursor varied, according to the concentration of FCS in the culture medium, used to maintain Sertoli cells in culture. Thus, in the absence of FCS (0%), $^{35}SO_4$ incorporation was low (44,000 cpm/1 × 10⁶ cells), while in 1% and 10% FCS, the incorporation of the precursor was 1.7 and 4.5 times higher (Table II).

To determine whether the increased incorporation of $^{35}SO_4$ into MA-PG caused by FCS correlated with changes in the net charge density of PG, MA-PG synthesized by Sertoli cells under different FCS concentration were resolved by DEAE–Sephacel chromatography.

As shown in Figure 4, changes in the concentration of FCS in the culture medium had several results. First, changes in the total radioactivity of PG increased linearly with increases in FCS concentrations. Second, a modification in the ratio of radioactivity was associated with mixed and CS-PG. At low serum concentration (0% and 1%) m-PG was more abundant than the CS-PG (ratio 1.3). This situation changed, how-

TABLE I. Characteristics of MA-PG Synthesized by Sertoli Cells

| | Chromatography | | | Sensitivity to[a] | | |
| | DEAE | CL-6B | | | | |
MA-PG	NaCl (M)	PG (K_{av})	GAG (K_{av})	CSase (%)	HNO₂ (%)	GAG chain
m-PG	0.35	0.48	0.55	65	35	HS,CS
CS-PG	0.45	0.35	0.55	100	0	CS

[a]Percentage of $^{35}SO_4$-labeled molecules sensitive to chondroitinase ABC/AC II (CSase) and nitrous acid treatment. m-PG: mixed proteoglycan; CS-PG: chondroitin sulfate proteoglycan; HS, CS, heparan sulfate and chondroitin sulfate glycosaminoglycan chains.

TABLE II. Chromatographic Characteristics of MA-PG Synthesized by Sertoli Cells at Different FCS Concentrations

		Chromatography					
		DEAE		CL-6B			
				PG		GAG from	
FCS (%)	$^{35}SO_4$[a]	m-PG	CS-PG	m-PG	CS-PG	m-PG	CS-PG
		NaCl (M)		(K_{av})			
0	1.0	0.30	0.37	0.48	0.35	0.55	0.45
1	1.7	0.35	0.42	0.48	0.35	0.55	0.45
10	4.5	0.35	0.45	0.48	0.35	0.55	0.45

[a]Relative incorporation of $^{35}SO_4$ into MA-PG. Incorporation at 0% FCS corresponds to 44,950 cpm/1 × 10⁶ cells.

ever, when Sertoli cells were cultured in the presence of 10% FCS. Under this condition, cells synthesized more CS-PG than m-PG (ratio 0.45). Third, a shift in the elution profile of each component. Thus, at 0% FCS, m-PG eluted from the DEAE–Sephacel column at 0.30 M NaCl, while at 1% and 10% FCS, the elution profile was shifted to 0.35 M NaCl. On the other hand, at 0% FCS, CS-PG, which eluted from the DEAE–Sephacel column at 0.37 M, changed its elution profile to 0.42 M and 0.45 M NaCl, when cells were incubated at 1% and 10% FCS, respectively.

These results demonstrate that MA-PG synthesized by Sertoli cells at high serum concentration (10%) exhibit a negative charge density higher than that exhibited by PG synthesized at low FCS concentrations.

Gel filtration studies were performed to analyze whether the FCS-dependent incorporation of $^{35}SO_4$ into MA-PG synthesized was related to changes in the molecular size of each PG and/or their GAG chains.

The results in Table II show that both mixed and CS-PG did not change their K_{av} in Sepharose CL-6B, as cells were cultured at 0%, 1%, or 10% FCS. In all conditions, m-PG eluted with a K_{av} of 0.48 and CS-PG with a K_{av} of 0.35. Similarly, at all FCS concentrations, GAG chains isolated from m-PG eluted at a K_{av} = 0.55 and those derived from the CS-PG eluted at K_{av} = 0.45.

As evidenced by enzymatic and chemical treatments, the chemical nature of MA-PG was not modified by changes in FCS concentrations. At all FCS concentrations, the sensitivity of m-PG
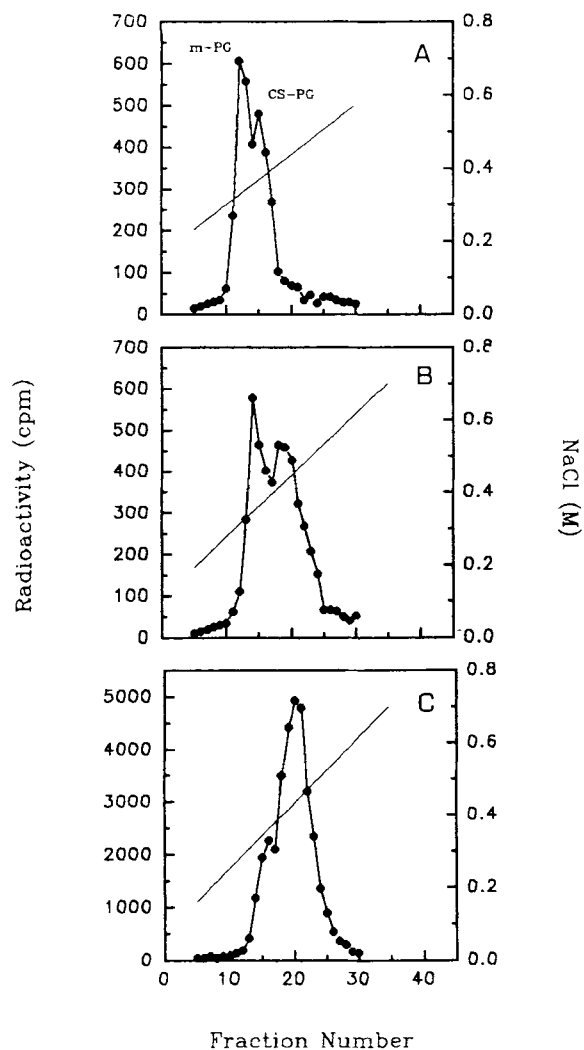
Fig. 4. DEAE–Sephacel chromatography of MA-PG produced by Sertoli cells at 0% (A), 1% (B), and 10% (C). After elution, aliquots of column fractions were taken for radioactivity and sodium chloride content measurements.

and CS-PG to chondroitinase ABC and nitrous acid treatments was similar to that shown in Table I.

Thus, the differences observed in the salt concentration required to elute MA-PG synthesized at different FCS concentrations from the DEAE–Sephacel column (Fig. 4) cannot be explained in terms of changes in the hydrodynamic size and chemical nature of each PG or its constituent GAG chains. These results are suggestive of FCS-dependent changes in the sulfation extent of MA-PG.

Na$_2$^{35}SO$_4$ and ^{3}H-glucosamine double-labeling. To further substantive FCS increases,

proteoglycan sulfation, Sertoli cells, at different FCS concentrations, were double labeled with ^{35}SO$_4$ and ^{3}H-glucosamine, and the labeled molecules were subsequently separated by DEAE–Sephacel chromatography, and the ^{35}SO$_4$/^{3}H ratio incorporated into MA-PG calculated.

The analysis of each radioactive precursor incorporated into the MA-PG from Sertoli cells cultured at different FCS concentrations is presented in Table III. The incorporation of each precursor into MA-PG increased as FCS concentration was modified from 0 to 10%. The ratio of radioisotopes (^{35}SO$_4$/^{3}H) incorporated into MA-PG increased from 17.6 in MA-PG synthesized at 0% FCS to 23.6 and to 50.9 in MA-PG synthesized by Sertoli cells cultured at 1% and 10% FCS, respectively.

These results demonstrate that as the FCS concentration increases, MA-PG produced by Sertoli cells become more sulfated.

DISCUSSION

This work demonstrates that MA-PG synthesized by Sertoli cells modify their structural characteristics according to changes in the culture conditions. Indeed, changes in the FCS concentration modulate the synthesis, as well the structural characteristics of the MA-PG produced by Sertoli cells in culture. In fact, when Sertoli cells are cultured in a medium supplemented with 10% FCS, the incorporation of sulfate into both MA-PG was higher than the incorporation observed at low serum (1% or none). Under all FCS conditions neither the hydrodynamic size of each MA-PG nor the hydrodynamic size of the GAG chains released from each MA-PG was modified.

However, the elution profiles in ion-exchange chromatography of each MA-PG varied according to changes in the FCS concentration. Thus,

TABLE III. Double Labeling of MA-PG Produced by Sertoli Cells at Different FCS Concentrations

FCS (%)	Incorporation[a]		Ratio
	^{35}SO$_4$	^{3}H-Glucosamine	^{35}SO$_4$/^{3}H
	(cpm/1 × 10^6 cells)		
0	44,950	2,550	17.6
1	77,900	3,300	23.6
10	203,600	4,000	50.9

[a]Single isotope incorporation was obtained from the radioactivity recovered after DEAE–Sephacel chromatography.

in the presence of serum (1% or 10%) both mixed and CS-PG show a higher charge density than that of the corresponding PG species synthesized at 0% FCS. This is shown by the high salt concentration required to elute each component from the DEAE–Sephacel columns.

Thus, changes in the FCS concentration seem to modify the extent of sulfation of the GAG chains in MA-PG. This was confirmed by results of double-labeled experiments using $^{35}SO_4$ and ^3H-glucosamine as GAG precursors. Although FCS affects both MA-PG species, the effect seems to be more pronounced in CS-PG than m-PG. This is revealed by the occurence of an increased $^{35}SO_4$ incorporation together with a pronounced shift to the left in the elution profile from the ion-exchange chromatography.

Changes in the sulfation of PG molecules have been reported in several other cell systems. In skeletal muscle cell cultures, developmental changes in the sulfation extent of CS-PG have been demonstrated [Hutchison and Yasin, 1986]. Also, the sulfation of HS-PG synthesized by transformed mouse mammary epithelial cells is lower than the HS-PG synthesized by normal cell cultures [David and Van Den Berghe, 1983]. Similarly, HS-PG produced by hepatoma cells are less sulfated and exhibit a lower average charge density than that of the HS-PG synthesized by normal liver cells [Robinson et al., 1984]. Also, changes in the sulfation extent of the HS-PG produced by hepatocytes are related to cell proliferation [Fedarko and Conrad, 1986].

In cultured Sertoli cells, changes in the synthesis of mixed and CS-PG are related to the age and the sexual development of the animals [Rodríguez and Minguell, 1989]. In addition, the synthesis of MA-PG by these cells is increased when they are plated onto a collagen substrate [Rodríguez and Minguell, 1992b]. This indicates that Sertoli cell–ECM molecules interactions play a role in Sertoli cell function.

It is known that the usual mode of binding of PG to other matrix molecules occurs through interaction of the GAG component with a binding site in the matrix protein. Since proteoglycans are known to bind to fibronectin, collagen, and laminin, it was postulated that MA-PG might be the receptors for matrix assembly [Ruoslahti, 1988]. Thus, changes in the amount as well as the molecular characteristics of MA-PG may conduct to changes in the physiology of the Sertoli cells.

Changes in the amount and/or in the charge density of MA-PG of Sertoli cells may modify their interactions to ECM components and germinal cells. These interactions are known to determine the morphological and physiological features of Sertoli cells, as well as their polarized pattern. In fact, the polarized pattern is related to the release of androgen-binding protein (ABP) and transferrin to the extracellular medium [Hadley et al., 1987; Janecki and Steinberger, 1986, 1987]. The interaction of Sertoli cells with ECM components, via MA-PG, results in an increased responsiveness to FSH [Tung and Fritz, 1984], which in turn improves their interaction with the germinal cells. This latter fact enhances the survival of the spermatogenic cells [Hadley et al., 1985; Tung and Fritz, 1984].

The fact that FCS modify the structure of MA-PG produced by Sertoli cells in vitro may be an important finding related to modulation of Sertoli cell physiology. It would be interesting to know what factor(s) present in the FCS modulate the sulfation of PG in this important cells. This area is currently under investigation in our laboratory.

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