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METABOLISM OF GLYCOSAMINOGLYCANS OF CULTURED RAT AORTIC SMOOTH MUSCLE CELLS ALTERED DURING SUBCULTURE

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Summary.—We observed the changes in the metabolism of glycosaminoglycans of cultured smooth muscle cells from the rat aorta during subculture. The primary culture was achieved by the enzymatic dispersion method. The metabolism of glycosaminoglycans in smooth muscle cells was estimated by measuring the incorporation rate of D-[1-^{14}C] glucosamine and [^{35}S] sulphate. Smooth muscle cells were harvested by trypsinization, and glycosaminoglycans were separately extracted and purified from trypsin digest and cells. In the cells of the stationary phase of primary culture, the incorporation of both D-[1-^{14}C] glucosamine and [^{35}S] sulphate into dermatan sulphate was greater than that into heparan sulphate. However, in the trypsin digest, the incorporation of D-[1-^{14}C] glucosamine and [^{35}S] sulphate into dermatan sulphate was less than and equal to that into heparan sulphate. In both the cells and the trypsin digest, the incorporation of D-[1-^{14}C] glucosamine and [^{35}S] sulphate into heparan sulphate decreased and that into dermatan sulphate increased with increase in the number of passages. These results indicate that there is a development of serial modulation in the metabolism of glycosaminoglycans in the cultured smooth muscle cells, in the early passage of subculture and that such should be taken into consideration when analysing the observations.

PROTEOGLYCANS and their constituent glycosaminoglycans (GAGs) are components of the arterial wall (Gore and Larkey, 1960) and quantitative and compositional changes in these macromolecules occur during the development of atherosclerosis (Iverius, 1972; Kumar *et al.*, 1967; Nakamura *et al.*, 1971). GAGs are synthesized and secreted by smooth muscle cells (SMCs), both in *vivo* and in culture (Wight and Ross, 1975*a*; Wight and Ross, 1975*b*).

Cultured aortic SMCs have been used in studies on atherogenesis (Chamley-Campbell, Campbell and Ross, 1979), in which subcultured SMCs from the explant migrated cells (Ross, 1971) were mainly used, probably because of the availability of a large quantity. However, to what extent the subcultured SMCs maintained their original and phenotypic characteristics of SMCs was unknown. Chamley *et al.* (1977) developed a culture method of the enzymatically dispersed SMCs from the aorta, which made feasible the procurement of the native contractile phenotype of SMCs in primary culture, and phenotypic modulation of SMCs occurring during subculture could be studied. In the present study, the arterial SMCs of the native phenotype were obtained in primary culture by the method of Chamley *et al.* (1977) and sequential changes in the metabolism of GAGs during subculture of SMCs were investigated.

MATERIALS AND METHODS

Cell culture.—Rat aortic smooth muscle cells (SMCs) were cultured by the method of Chamley *et al.* (1977) with minor modification. Briefly, the aortas were excised from male Wistar rats (200–250g) and the adventitia and the intima were stripped off after treatment with collagenase

and elastase. The media thus obtained was dispersed into single cells and cell clumps by a second incubation with collagenase and elastase. The dye exclusion test (Phillips, 1973) indicated high, nearly 98%, viability of the dispersed cells. The cells were grown in plastic dishes (Falcon; Becton, Dickinson and Company, Oxnard. CA) of 60 mm diameter containing 4 ml of Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% foetal calf serum, 100 μg/ml streptomycin and 100 u/ml penicillin. The medium was changed every 3 days and the cells were subcultured every 7–9 days (1:2 split) after trypsinization. Eleven cell lines of SMCs up to the 12th passage were maintained and examined in the present study. Where indicated, the fresh floating medial explants of rat aorta were used and were prepared by the method of Jarmolych *et al.* (1968).

Morphological examination.—Morphological characteristics of SMCs in primary culture and subculture were examined under an electron microscope and by direct immunofluorescent staining of smooth muscle actin and myosin. For direct immunofluorescent staining, smooth muscle actin and myosin were prepared by the methods of Spudich and Watt (1971) and Sobieszek and Small (1976) from a chicken gizzard, respectively. The antiactin antiserum and antimyosin antiserum were prepared in rabbits, and the IgG fractions of antisera were isolated and conjugated with fluorescein isothiocyanate (FITC) (Kawamura, 1976). The antibody solution had an appropriate FITC protein ratio of 1:2. The cells fixed with cold 95% ethanol for 30 min were stained with FITC conjugated antibody solution for 3 h at a room temperature of 25°. For electron microscopic examination, SMCs were fixed in 3% glutaraldehyde in 0·2 M cacodylate buffer at pH 7·4 for 2 h, post-fixed in 1% osmium tetroxide for 2 h and dehydrated and embedded in Epon 812. The thin sections of the samples were stained with lead citrate and examined with a Hitachi HS7D transmission electron microscope (Hitachi Co., Tokyo).

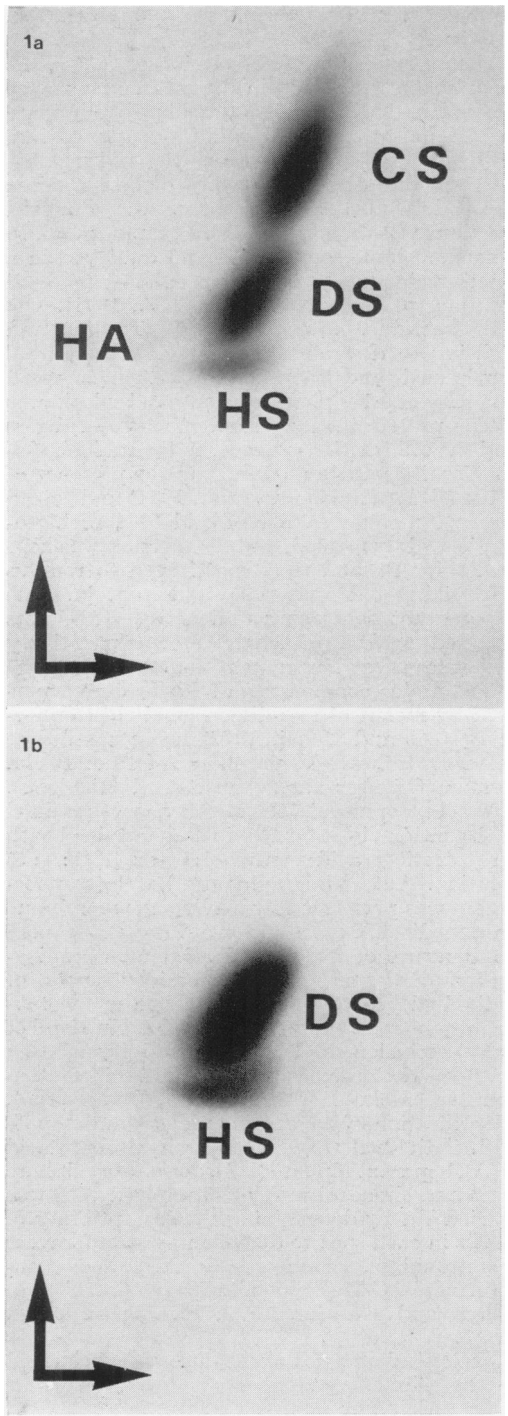
Study on the metabolism of GAGs in SMCs.—Cultured SMCs of the desired passage number were incubated in a growth medium containing either 5 μCi/ml sodium [^{35}S] sulphate or 2 μCi/ml D-[1-^{14}C] glucosamine (New England Nuclear, Boston, Mass.) at 37° in a CO_2 incubator. After incubation for the desired time, the growth medium was decanted and stored at −20°, as the medium fraction. The cell layer was rinsed 5 times with 4 ml of Hanks' solution and treated with 4 ml of trypsin (200 u/ml) and EDTA (0·01%) in Dulbecco's phosphate buffered saline (PBS) at 37° for 7 min. The cell layer was dissociated quickly from the dish by pipetting and immediately cooled on ice. The resultant cell suspension was centrifuged (800 *g*, 10 min) at 4°, and the supernatant was decanted and stored at −20° in a freezer as the trypsin digest fraction. The precipitate was washed once with Dulbecco's PBS and stored at −20° as the cell fraction.

Before extraction and isolation of GAGs, the medium and the trypsin digest fractions were thawed and dialysed thoroughly to eliminate free radioisotopes. For studies using sodium [^{35}S]sulphate, samples were dialysed against 0·01 M sodium sulphate before dialysis against distilled water. Subsequently, the dialysed fractions were concentrated by evaporation into a small volume, lyophilized and dissolved in a small volume of 0·1 M phosphate buffered solution (pH 6·5) (Nakamura *et al.*, 1971). The cell fractions, after repeated freezing and thawing to destroy the cell membranes, were lyophilized and then suspended in a small quantity of 0·1 M phosphate buffered solution.

The protein in the media, the trypsin digest and the cell fractions were then digested for 48 h at 60° with papain (20 u/ml), in the presence of 0·1 M EDTA (Nakemura *et al.*, 1971) and cooled on ice. An equal volume of cold 10% trichloroacetic acid was added, and the mixture was kept on ice for 1 h and then centrifuged to remove the undigested materials and protein. The supernatant containing radioactive GAGs was dialysed against distilled water exhaustively and concentrated by evaporation.

The major components of GAGs were separated by means of two-dimensional electrophoresis (Hata and Nagai, 1972) using a cellulose acetate membrane (Sepraphore III 10 × 10 cm, Gelman Sciences Inc., Ann Arbor, Michigan). After electrophoresis, the membrane was stained with gentian violet for 15 min and destained with an acetic acid:methanol:water (1:4:5) solution. Autoradiography of the electrophorogram was performed by leaving the membrane on a medical X-ray film for 5–7 days at a room temperature of 25° (Fig. 1). After autoradiography, the gentian violet stained spots of individual GAGs on the cellulose acetate membrane were cut out and dissolved in 10 ml of Bray's solution, and the radioactivity of each spot was determined using a liquid scintillation counter (Aloka LSC-700, Aloka, Co., Tokyo). Identification of the gentian violet stained spots with individual GAGs such as hyaluronic acid (HA), heparan sulphate (HS), dermatan sulphate (DS) and chondroitin -4 or -6 sulphate (CS) was carried out by determining the susceptibility of GAGs in each spot to digestion by streptomyces hyaluronidase, chondroitinase ABC and chondroitinase AC-II (Seikagaku Kogyo Co., Tokyo) (Ohya and Kaneko, 1970; Yamagata *et al.*, 1968).

Statistical analysis of the data was performed by the paired *t* test.



RESULTS

Morphological observations

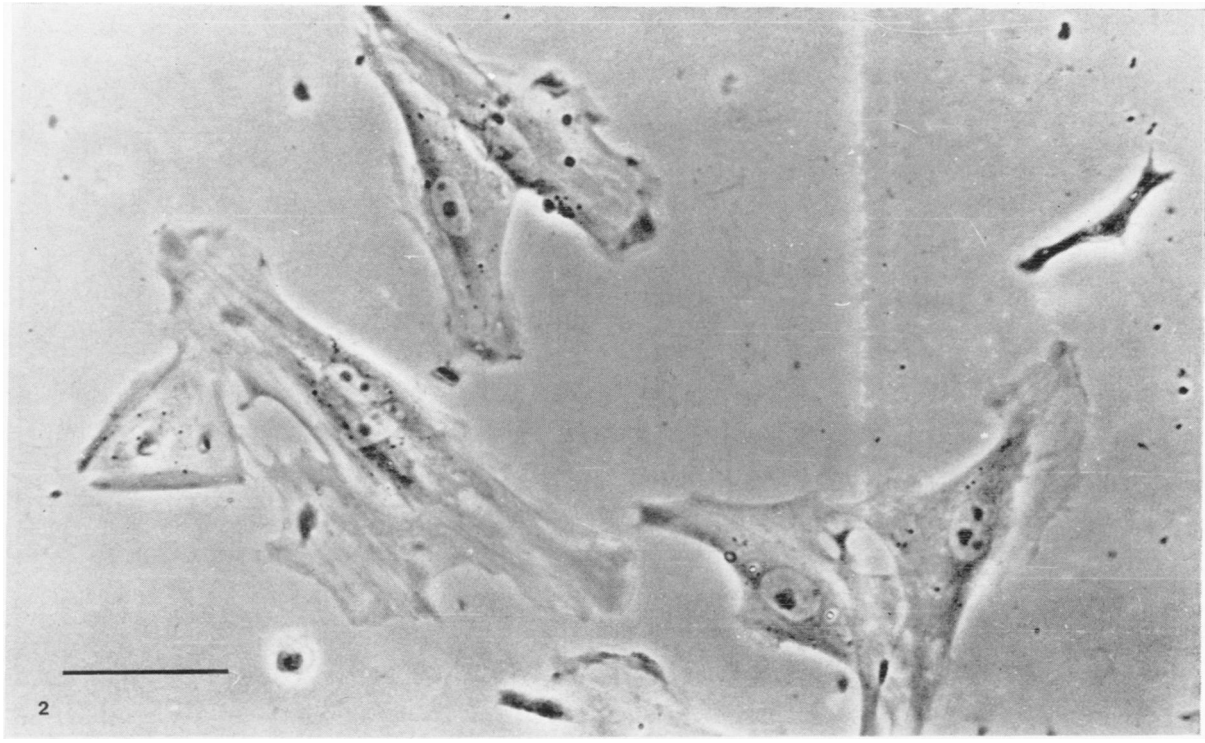
Phase contrast microscopic examinations revealed that the cells in primary culture obtained by the method of Chamley *et al.* (1977) were exclusively one cell type characterized by the ribbon or spindle shape and phase dense cytoplasm containing filamentous structure parallel to the longitudinal axis of the cells (Fig. 2). Electron microscopic examinations revealed the presence of large numbers of thin filaments in the cytoplasm and substances that resembled the basement membrane in the intercellular space (Fig. 3). We indicated that the cells in primary culture possessed morphological characteristics of SMCs (Pease and Paule, 1960).

Direct immunofluorescent staining of actin showed positive staining in cultured SMCs throughout the serial passages up to the 12th subculture; however, the intensity of the fluorescence decreased with the number of passages (Fig. 4a). On the other hand, myosin was detected only in the primary culture and in the second passage by immunofluorescent staining (Fig. 4b).

Incorporation of [^{35}S] sulphate into GAGs in SMCs

The time-dependent incorporation of [^{35}S]sulphate into GAGs in the medium, the trypsin digest and the cell fractions of SMCs in the third passage shown in Fig. 5 are all but identical to those of the 6th passage. The increment of incorporation of radioactive GAGs into the medium and the trypsin digest fractions indicated that GAGs synthesized in SMCs (the cell fraction) might be transferred to the surface-associated pool (the trypsin digest

FIG. 1.—Two-dimensional electrophorogram of GAGs extracted from the cell fraction of the rat aortic SMCs in primary culture. (a) shows the gention violet stained electrophorogram of GAGs of the cell fraction of SMCs treated with [^{35}S] sulphate and (b) its autoradiogram. Horizontal and vertical arrows indicate the 1st and 2nd directions of electrophoresis, respectively. HA: hyaluronic acid, HS: heparan sulphate, DS: dermatan sulphate and CS: chondroitin -4 or -6 sulphate.



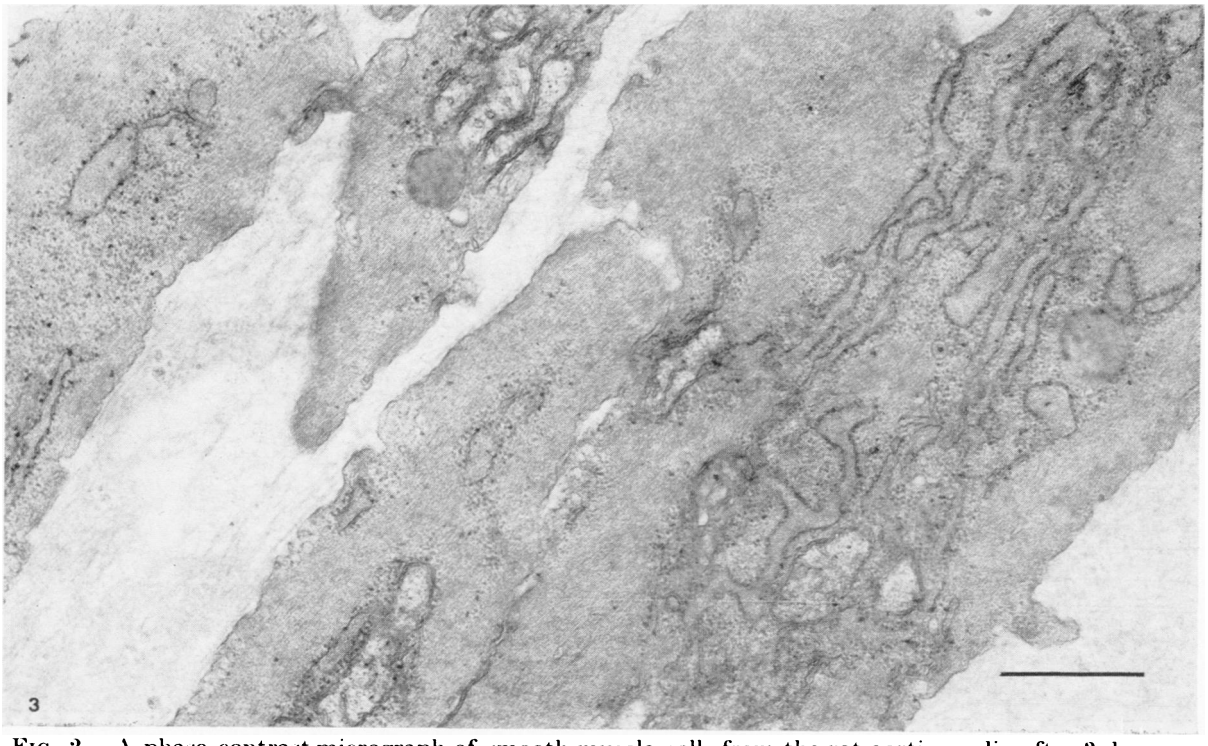


FIG. 2.—A phase contrast micrograph of smooth muscle cells from the rat aortic media after 3 days in primary culture. Bar, 50 μm. $\times$ 376.

FIG. 3.—An electron micrograph of a smooth muscle cell from the rat aortic media after 12 days in primary culture. The cytoplasm is largely filled with thin filaments, and basement membrane-like substances are evident in the intercellular space. Bar, 1 μm. $\times$ 16,000.

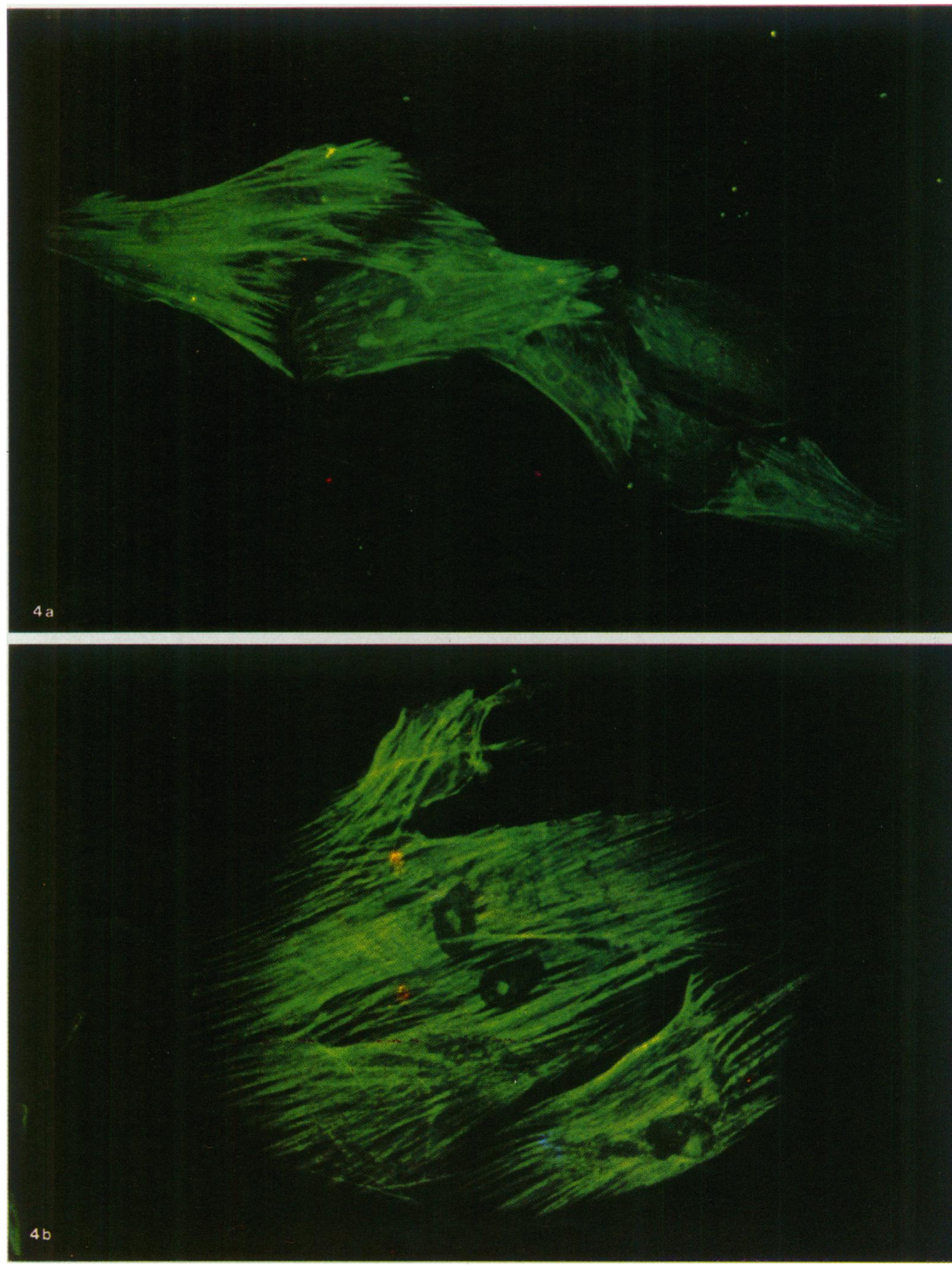


FIG. 4.—(a) Smooth muscle cells in the 5th passage stained with FITC labelled antibodies against smooth muscle actin. × 275. (b) Smooth muscle cells in primary culture stained with FITC-labelled antibodies against smooth muscle myosin. × 440.

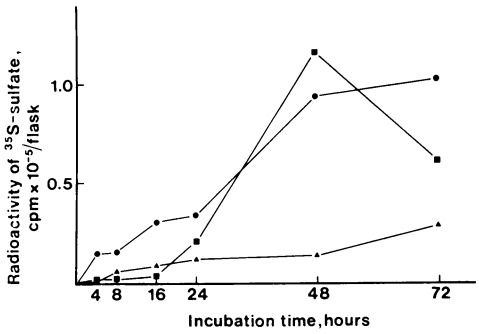


FIG. 5.—Incorporation of [^{35}S] sulphate into GAGs of the medium (●), the trypsin digest (▲), and the cell fraction (■) of SMCs in the 3rd passage. Cells were incubated with 5 μCi/ml of sodium [^{35}S] sulphate for the desired interval indicated on the abscissa. Incorporated radioactivity was expressed as ct/min/flask (Namiki *et al.*, 1980).

fraction) and to the extracellular pool (the medium fraction) because the latter 2 pools had no synthetic system for GAGs. The amount of radioactivity incorporated into each pool reached the maximum level at 48 h after incubation. Therefore, the incubation time was fixed at 48 h in the subsequent experiments.

Distribution of radioisotopes into individual components of GAGs

The percent distribution of D-[1-^{14}C] glucosamine into individual GAG components of SMCs in lag and stationary phases of the primary culture is shown in Table I. For a comparison, the percent distribution of radioactivity in the fresh floating explants of the initial 2 culture days and which were treated with D-[1-^{14}C] glucosamine and processed similarly to enzymatically dispersed cells of primary culture, are also listed in Table I. In case of the floating medial explants, D-[1-^{14}C] glucosamine was incorporated mainly into HA and DS. In the lag phase of the primary enzymatic dispersion culture, the radioactivity of D-[1-^{14}C] glucosamine in the cell fraction was recovered mainly in HA, and that recovered in DS was low. In the stationary phase of SMCs in primary culture, D-[1-^{14}C] glucosamine was incorporated mainly into HS and DS. There was a significant difference in the relative distribution of radioactivity between the cell fraction and the trypsin digest fraction; the incorporation of radioactivity into HS was significantly higher ($P < 0.05$) in the trypsin digest fraction than in the cell fraction.

Modulation of the synthesis and secretion of GAGs in the stationary phase of various passages of subcultured SMCs

The distribution of D-[1-^{14}C] glucosamine and [^{35}S] sulphate into components of GAGs of the cell fraction and the trypsin digest fraction of SMCs in primary culture and in the 3rd, 5th, 7th and 10th passages are shown in Table II. GAGs of the medium fraction were not examined in detail because of the difficulty in purification. In the cell fraction of SMCs in

TABLE I.—*Incorporation of D-[1-^{14}C] glucosamine into GAGs of SMCs of floating explant culture and primary cell dispersion culture of lag phase and stationary phase*

	HA	HS	DS	CS
Floating explant (N = 5)	50·0 ± 9·1	13·3 ± 3·2	33·3 ± 4·1	3·4 ± 2·2
Enzymatic cell dispersion culture lag phase (N = 3)	70.2 ± 6·1	13·0 ± 2·2	11·7 ± 3·9	5·1 ± 0·8
Stationary phase (N = 5)				
cell fraction	8·9 ± 3·8	33·9 ± 21·7	54·9 ± 22·4	2·3 ± 0·9
trypsin digest fraction	10.4 ± 5·6	59·0 ± 8·6*	24·2 ± 6·1**	6·4 ± 4·2

Rat aortic SMCs or explants were incubated in the presence of 2 μCi/ml D-[1-^{14}C] glucosamine for 48 h and processed as described in Materials and Methods. The values for individual GAGs are expressed as percent distribution of D-[1-^{14}C] glucosamine radioactivity (mean ± s.d.). For the stationary phase of enzymatic cell dispersion culture, the values of individual GAGs in the trypsin digest fraction were compared with those in the cell fraction. *$P < 0.05$, **$P < 0.025$, N = number of experiments.

TABLE II.—*Percent distribution of D-[1-^{14}C] glucasamine and [^{35}S] sulphate in GAGs in both the cell fraction and the trypsin digest fraction of cultured SMCs in primary culture and subcultures*

Cell fraction (intracellular glycosaminoglycans)

Passage number	Tracer: D-[1-^{14}C] glucosamine (N = 5)					[^{35}S] sulphate (N = 6)				
	1	3	5	7	10	1	3	5	7	10
HA	8·9±3·8	7·2±5·2	7·2±4·8	5·0±2·4	3·2±2·7	—	—	—	—	—
HS	33·9±21·7	30·5±16·8	18·1±7·3	10·2±4·9*	6·8±2·4*	38·8±16·3	25·2±11·8*	27·0±13·3	15·5±6·6*	9·1±2·1***
DS	54·9±22·4	61·0±17·8	73·1±8·0	81·0±5·6**	87·7±3·5**	55·6±13·4	64·9±12·0	65·6±9·9	81·0±10·0*	84·1±5·3****
CS	2·3±0·9	1·3±1·2	1·6±1·0	3·8±3·3	2·2±1·0	5·6±5·5	9·9±4·5	7·4±4·1	3·5±4·1	6·8±3·5

Trypsin digest fraction (intercellular glycosaminoglycans)

Passage number	Tracer: D-[1-^{14}C] glucosamine (N = 5)					[^{35}S] sulphate (N = 6)				
	1	3	5	7	10	1	3	5	7	10
HA	10·4±5·6	11·0±7·0	12·4±9·6	10·2±7·8	18·5±13·4	—	—	—	—	—
HS	59·0±8·6	57·4±10·3	62·2±11·6	43·9±11·0**	36·9±12·4**	47·6±10·2	50·2±15·6	54·8±2·3	49·2±16·2	40·7±7·1
DS	24·2±6·1	29·7±7·9	21·3±4·1	43·4±11·0**	42·2±11·8**	47·1±8·1	44·2±14·4	41·3±4·2	46·5±14·3	56·0±7·7
CS	6·4±4·2	1·9±1·3	4·1±4·1	2·5±2·3	2·4±1·8	5·3±3·2	5·6±2·4	3·9±2·9	4·3±2·6	3·3±1·4

Rat aortic SMCs in stationary phase of primary culture or subculture were incubated with 5 μCi/ml sodium [^{35}S] sulphate or 2 μCi/ml D-[1-^{14}C] glucosamine for 48 h and processed as described in Materials and Methods. The sum of the radio-active GAGs isolated from the different pools is defined as 100%. The values of individual GAGs in subcultures are compared with those in primary cultures. *$P < 0·05$, **$P < 0·025$, ***$P < 0·01$, ****$P < 0·005$. Mean ±s.d. N = number of cell lines.

primary culture, D-[1-^{14}C] glucosamine was incorporated mainly into HA, HS and DS. The incorporation of D-[1-^{14}C] glucosamine into DS in the 7th and 10th passages was significantly greater ($P < 0{\cdot}025$), and that into HS was significantly less ($P < 0{\cdot}05$) than in the primary culture, indicating that as the passage number of subcultures increased, the distribution of the incorporation of D-[1-^{14}C] glucosamine into DS increased and that into HS decreased. In the cell fraction, [^{35}S] sulphate was incorporated mainly into HS and DS (Fig. 1b, Table II), and the distribution of radioactivities into those 2 components of GAGs serially and significantly changed with increase in number of passages of subcultures, as was observed in the incorporation of D-[1-^{14}C] glucosamine.

In the trypsin digest fraction of SMCs in primary culture, D-[1-^{14}C] glucosamine was distributed mainly in HA, HS and DS. There was a significant increase in the distribution of radioactivity in DS in the 7th and 10th passages ($P < 0{\cdot}025$). Although the distribution of radioactivity in HS in the 7th and 10th passages was significantly less ($P < 0{\cdot}025$) than that in the primary culture, it was maintained at a relatively higher level than that of the cell fraction. When [^{35}S] sulphate was used as a substrate, the relative incorporation of radioactivity into DS tended to increase, that into HS tended to decrease, as the passage number of the subcultures increased.

DISCUSSION

Although synthesis of GAGs by cultured SMCs has been well examined using radioactive tracers (Deudon *et al.*, 1980; Gamse, Fromme and Hesse, 1978; Kresse *et al.*, 1975; Namiki *et al.*, 1980; Pietila *et al.*, 1980; Wight and Ross, 1975*b*), the component distribution of GAGs in cultured SMCs remains controversial. Such may be attributed to the differences in animal species and in the experimental conditions, in particular the isolation procedure of GAGs. In addition, it should be emphasized that, in all previous studies, the investigators used subcultured cells from various passage numbers and did not take into consideration phenotypic modulation of SMCs in culture. When cultured SMCs are being used, in particular in studies concerning atherogenesis, it seems important to determine whether these cultured cells are exclusively derived from SMCs and if they maintain phenotypic characteristics of SMCs which possess significant functions in atherogenesis, *in vivo* (Ross and Gromset, 1973).

Chamley *et al.* (1977) proposed that immunofluorescent staining of smooth muscle actin and myosin allowed for a correct identification of SMCs. They also emphasized the occurrence of phenotypic modulation of SMCs in culture during the proliferative stage of primary culture and in subcultured cells, and recommended parallel studies using cultured SMCs of mature, contractile and functional types as well as modulated, primitive and synthetic types (Chamley-Campbell *et al.*, 1979). In the present study we used the method of Chamley *et al.* (1977) for primary culture of rat aortic medial SMCs and confirmed the modulation of cultured cells using direct immunofluorescent staining of actin and myosin. SMCs after the 3rd passage had actin but no myosin, thereby suggesting that these cells after the 3rd passage were in a modulated primitive state of SMCs.

There was a difference between the enzymatic cell dispersion method and the floating explant culture method in the relative incorporation of D-[1-^{14}C] glucosamine into GAGs of SMCs in primary culture. There was also a difference in incorporation of D-[1-^{14}C] glucosamine between the lag phase and stationary phase of the cells in primary culture. These findings suggest that the culture method and the culture phase used greatly influence the metabolism of GAGs in SMCs.

In the primary culture, we noted the predominant distributions of D-[1-^{14}C] glucosamine and [^{35}S] sulphate in HS of the trypsin digest fraction, as compared with findings in the cell fraction, and which is in

agreement with the findings of Deudon *et al.* (1980).

The present study, in which the 11 cell lines of rat aortic SMCs were maintained up to the 12th passage, indicated that there was a modulation of GAGs metabolism in association with the phenotypic modulation related to the myosin content during subculture. The incorporation of both D-[1-^{14}C] glucosamine and [^{35}S] sulphate into HS decreased and that into DS increased gradually with increase in the number of passages, in both the cells and the trypsin digest fractions. An alteration of synthesis by the cells and of resultant secretion into the surface associated pool (the trypsin digest fraction) of GAGs seemed to be initiated in the 3rd passage of subculture, when initiation of phenotypic modulation of cultured SMCs occurred, as evidenced by the loss of myosin.

In conclusion, it has to be emphasized that the metabolism of GAGs in cultured SMCs is rather diversified, depending on the culture method employed, and undergoes various steps of modulation during the subculture. From this point of view, the culture method and the number of passages should be specified when using cultured SMCs. Further studies are underway to elucidate the functional significance of alteration of the metabolism of GAGs in SMCs during subculture.

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