Effects of glycosaminoglycans on proliferation of epithelial and fibroblast human malignant mesothelioma cells: a structure–function relationship

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Abstract. Proteoglycans interact with other effective macromolecules regulating a variety of cellular events via their glycosaminoglycan (GAG) chains. The effects of all known glycosaminoglycans (GAGs) produced by normal cells and tissues on the proliferation of two human malignant mesothelioma cell lines, one with fibroblast-like morphology and the other with epithelial differentiation – both able to produce hyaluronan (HA), galactosaminoglycans (GalAGs) and heparan sulphate (HS) containing proteoglycans – have been studied. Cell proliferation was assessed by measuring [³H]thymidine incorporation and cell number. GalAGs, i.e. chondroitin sulphates (CSs) and dermatan sulphate (DS), strongly stimulate the proliferation of fibroblast-like cells in a dose-dependent manner (170–250% at 100 µg/ml), independently of their sulphation pattern. In epithelial cells, however, only DS stimulates cell proliferation. The effects of CSs on proliferation of epithelial cells are not depended on their sulphation pattern. Thus, CSs either with $[-\text{GlcA-GalNAc-}(-6\text{-O-SO}_3^-)]$ - or $[-\text{GlcA-GalNAc-}(-4\text{-O-SO}_3^-)]$ - as the commonest unit, had no significant effect. L-Iduronic acid (IdoA)-rich heparin and fast-moving HS (fm-HS), a HS fraction with a heparin-like structure, had significant antiproliferative effects on mesothelioma cells of both types (30–70% at 1.0 µg/ml

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The following abbreviations are used throughout GlcA, D-glucuronic acid, GlcN, D-glucosamine, $\Delta\text{di-nonS}_{\text{HA}}$, 2-acetamido-2-deoxy-3-O-(4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid)-D-glucose, $\Delta\text{di-nonS}_{\text{CS}}$, 2-acetamido-2-deoxy-3-O-(4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid)-D-galactose, $\Delta\text{di-mono4S}$, $\Delta\text{di-mono6S}$ and $\Delta\text{di-monoNS}$, monosulphated Δ -disaccharides at C-4, C-6, and the amino group of hexosamine, respectively, $\Delta\text{di-mono2S}$, monosulphated Δ -disaccharides at C-2 of uronic acid, $\Delta\text{di-di}(2,4)\text{S}$ and $\Delta\text{di-di}(2,6)\text{S}$, disulphated Δ -disaccharides at C-2 of uronic acid and C-4 of hexosamine and at C-2 and C-6, respectively, $\Delta\text{di-di}(2,\text{N})\text{S}$ and $\Delta\text{di-di}(6,\text{N})\text{S}$, disulphated Δ -disaccharides at C-2 of uronic acid and the amino group of hexosamine and at C-6 and the amino group, respectively, $\Delta\text{di-tri}(2,4,6)\text{S}$, trisulphated Δ -disaccharides at C-2 of uronic acid and at C-4 and C-6 of hexosamine

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and 85–90% at 100 $\mu\text{g/ml}$, respectively). GlcA-rich HS, however, had no significant effects. HA inhibits only the proliferation of fibroblast-like cells by 25% at 50 and 100 $\mu\text{g/ml}$. Keratan sulphate suppresses cell proliferation (10–30%) in both cell lines. In the view of these findings, a structure–function relationship of GAGs on cell proliferation of the two human malignant mesothelioma cell lines is discussed. Other factors, such as chain conformation and geometry, as well as interactions of growth factors with GAGs, possibly involved in the regulation of cell proliferation, are also discussed.

INTRODUCTION

Proteoglycans (PGs) are macromolecules composed of glycosaminoglycan (GAG) and oligosaccharide chains covalently bound to a protein core. GAGs are linear polymers created by a repeating disaccharide unit containing hexosamine and uronic acid or galactose in the case of keratan sulphate (KS). Due to the various sulphate residues in the disaccharide units, PGs are highly charged macromolecules. They are found at both the extracellular and cellular (cell membrane and intracellular granules) levels (Kjellen & Lindhal 1991, David 1993). Through GAG chains, PGs interact with effector (structural, functional and regulatory) macromolecules, such as growth factors, growth factor receptors and extracellular matrix proteins; they participate in many biological events, such as blood coagulation, angiogenesis, metastasis, cell proliferation and differentiation (Yayon *et al.* 1991, Wight *et al.* 1992, Adams & Watt 1993, Laurent *et al.* 1996).

GAG constituents of PGs affect the proliferation of many cell lines *in vitro*. The effects and mechanisms of the L-iduronic acid (IdoA)-containing glucosaminoglycans (GlcAGs), i.e. heparin and heparan sulphate (HS), on cell proliferation have been extensively reviewed by Wright *et al.* (1989a). These GlcAGs inhibit the proliferation of several cells *in vitro*, such as smooth muscle cells (Castellot *et al.* 1981), mesangial (Castellot *et al.* 1985a), endothelial (Fritze *et al.* 1985) and epithelial cells (Wright *et al.* 1989b). Each one of these GlcAGs, however, may have different effects. Thus, heparin inhibits the proliferation of U-937 leukaemia cells (Volpi *et al.* 1994) and human lung fibroblasts (Westergren-Thorsson *et al.* 1993); HS has no significant effect on proliferation of these cells, whereas IdoA-rich HS inhibits cell proliferation to an extent similar to that of heparin (Wright *et al.* 1989b, Volpi *et al.* 1994). Although, no exact molecular mechanism by which these GAGs affect cell proliferation has yet been completely described, it seems that in transformed cell membrane receptors, growth factors, most probably produced by an autocrine pathway, and cell bound HSPGs play a main role (Wright *et al.* 1989a, Westergren-Thorsson *et al.* 1993). It has been proposed that heparin regulates proliferation, either by acting directly on cells through an internalization process via a receptor-mediated endocytosis or by an indirect mechanism via modulation of the activities of growth factors most likely produced by the cells themselves (autocrine mechanism) and not provided by the serum supplement (Kraemer 1971, Benitz *et al.* 1986, Wright & Karnovsky 1987, Westergren-Thorsson *et al.* 1993). The IdoA-containing GalAG, dermatan sulphate (DS), inhibits proliferation of human lung fibroblasts (Westergren-Thorsson *et al.* 1993), whereas it stimulates U-937 leukaemia cells significantly (Volpi *et al.* 1994). CS, a non-IdoA containing GalAG, is an effective stimulator of proliferation for both lung fibroblasts (Westergren-Thorsson *et al.* 1993) and leukaemia cells (Wright *et al.* 1989b). An extensive study by Volpi *et al.* (1993) on the effects of low and highly sulphated CSs on leukaemia cells showed that cell proliferation depends on the

composition of disulphated disaccharides in CS. Therefore the effect of GAGs on cell proliferation seems to be closely related to the structure of the GAG chain and on the cell type as well (Wright *et al* 1989a, Westergren-Thorsson *et al* 1993). Hyaluronan (HA) has been recognized as a potential marker to identify malignant mesothelioma in pleural effusion (Nurminen *et al* 1994). Recent studies revealed that HA receptors are expressed on human malignant mesothelioma cells but not on normal mesothelial cells and the HA production is closely related to the secretion of factors by normal mesothelial cells (Asplund & Heldin 1994). Very little is known about the influence of cell proliferation by other significant extracellular matrix macromolecules, such as KS and KS containing PGs.

Recently, we reported that human malignant mesothelioma cells produce HA and PGs bearing CS and HS chains (Klominek *et al* 1989, Tzanakakis *et al* 1995a). *In vitro* studies on two human malignant mesothelioma cell lines, one with a fibroblast-like morphology and the other with an epithelial phenotype, revealed that the synthesis and distribution of PGs in the cell membrane and the extracellular medium are highly dependent on cell phenotype and closely related to the effects of various growth factors (Tzanakakis *et al* 1995, Tzanakakis *et al* 1996). We therefore found it of great interest to characterize in great detail well-defined GAGs produced by normal cells and tissues and relate their structure with the proliferation rates of malignant mesothelioma cells. In this paper, we report the effects of GAGs and the relationship of their fine disaccharide composition to the proliferation of two human malignant mesothelioma cell lines.

MATERIALS AND METHODS

Materials

Chondroitin sulphate type A (CSA) (mainly C-4-sulphated CS) from bovine trachea, CSB (DS) from porcine skin, chondroitin type C (CSC) (mainly C-6-sulphated CS) from shark cartilage, heparin from porcine intestinal mucosa, fast-moving heparan sulphate (fm-HS) from bovine intestinal mucosa, HS from bovine kidney, KS from bovine cornea and heparin lyase II (heparinase II, no EC number) from *Flavobacterium heparinum* were obtained from Sigma Chemical Co (St Louis, MO, USA). HA (Healon) was purchased by Pharmacia (Uppsala, Sweden). Standard preparations of HA, CS/DS and HS-derived disaccharides, i.e. Δ di-nonS_{HA}, Δ di-nonS_{CS}, Δ di-nonS_{HS}, Δ di-mono4S, Δ di-mono6S, Δ di-mono2S, Δ di-di(2,4)S (Δ di-diS_B), Δ di-di(2,6)S (Δ di-diS_D), Δ di-di(4,6)S (Δ di-diS_E) and Δ di-tri(2,4,6)S (Δ di-triS) were purchased from the Seikagaku Kogyo Co (Tokyo, Japan). Chondroitinases ABC (EC 4.2.2.4), AC (EC 4.2.2.5) and B (no EC number) and chondro-4- (EC 3.1.6.9) and -6-sulphatase (EC 3.1.6.10), heparin lyase I (heparinase, EC 4.2.2.7) and heparin lyase III (heparitinase, EC 4.2.2.8) were also purchased from Seikagaku. Fetal calf serum (FCS), RPMI 1640-based medium, penicillin, streptomycin, gentamicin and L-glutamine came from Gibco (Grand Island, NY). Human AB serum and Hanks' balanced solution were purchased from Sigma. [³H]Thymidine was obtained from Du Pont de Nemours (Dreieich, Germany). All other chemicals used were of the best commercially available grade.

Cell cultures

Two human malignant mesothelioma cell lines one with epithelial (STAV-AB) and the other with fibroblast-like phenotype (STAV-FCS) established by Klominek *et al* 1989, kindly provided by Dr Klominek (Department of Lung Medicine, Karolinska Institute, Huddinge University Hospital, Huddinge, Sweden) were used in this study. Cells were grown on RPMI

1640-based medium supplemented by 10% (v/v) FCS for the STAV-FCS cell line and 10% (v/v) human AB serum for the STAV-AB cell line in the presence of 4 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 20 µg/ml gentamicin. Cultures were performed in 75-mm² tissue culture flasks in a humidified atmosphere of 5% (v/v) CO₂ at 37°C and the culture medium was changed every other day. Confluent cultures, after being washed with phosphate buffered saline (PBS), were harvested by trypsinization with 0.25% (w/v) trypsin in PBS containing 0.1% (w/v) Na₂EDTA for 15 min at 37°C and 1 volume of medium was added to terminate enzymatic activity. Cells were collected by centrifugation at 200 g for 5 min, and their number was measured by suspension in Hanks' balanced solution, using a Coulter particle-counter (Hialeah, FL, USA). Cells, 1 ml suspension with a density of 5×10^5 cells/ml and plate, were plated in 35-mm tissue culture dishes and cultured in serum-free RPMI-1640 based medium for 1–4 days, in the presence or absence of various GAGs at concentrations ranging from 0.01 to 100 µg/ml. GAGs in the form of Na⁺ salt before addition to culture were solubilized in RPMI and filtered on sterile membrane filters of 0.2-µm pore size (Millipore, Milford, MA, USA). Cell viability was determined by trypan blue dye exclusion.

Determination of the fine chemical composition of GAGs

Disaccharide composition

The disaccharide composition of HA, CSA, DS and CSC was determined by ion-suppression high-performance liquid chromatography (HPLC) and reversed polarity high-performance capillary electrophoresis (HPCE) methods (Karamanos *et al.* 1994, 1995). In brief, GAGs were dissolved in 50 mM Tris-HCl, pH 7.6, and digested with chondroitinases ABC or AC using 0.1 units/ml per g uronic acid at 37 °C for 90 min. The Δ-disaccharides obtained were separated by HPLC on an Econosphere-NH₂ (Alltech, IL, USA) column (240 × 4.6 mm i.d.) equipped with an amino (Brownlee, Alltech) precolumn (3 × 4.6 mm i.d.) and by HPCE on an uncoated fused silica capillary (Beckman, Nyon, Switzerland) and recorded at 232 nm. The disaccharide composition of heparin, HS and fm-HS was estimated by using both the ion-pair HPLC and HPCE methods (Karamanos *et al.* 1996a, 1997). These GAGs were degraded by an equi-unit mixture of heparin lyases I, II and III (0.05 units per 25 µg of uronic acid) in 20 mM acetate buffer, pH 7.0, containing 1 µmol calcium acetate at 37 °C for 90 min.

Determination of IdoA content

The IdoA to GlcA molar ratios were determined by the reversed phase HPLC method of Karamanos *et al.* (1988). GAGs were stoichiometrically reduced with NaBH₄ in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. Following acid hydrolysis of the quantitatively reduced GAGs (2 M trifluoroacetic acid, 100 °C, 8 h) and per-*O*-benzoylation of the obtained 1,6-anhydro-L-idose and D-glucose, the derivatives were purified through a Sep-Pak C-18 cartridge (Waters Associates, Milford, MA, USA) and separated on a Supelcosil LC-18 (Supelco Inc., Bellefonte, PA, USA) column (250 × 4.6 mm i.d.) eluted with 75% (v/v) aqueous acetonitrile at 1.0 ml/min. The peaks obtained were recorded at 230 nm.

Other analytical assays

N-Sulphated hexosamine was determined using the low-pH nitrous acid procedure (Shively & Conrad 1976) and *N*-acetylated hexosamine by the method of Tsuji *et al.* (1969). Relative

molecular mass (M_r) of GAGs was determined by HPLC, using a refractive index detection mode and separation in three serially connected μ -Bondagel E-500 columns, as previously described (Engfeldt *et al* 1982, Arvaniti *et al* 1994)

Biological assays

Determination of in vitro anticoagulation activity

The anticlotting activities of heparin, HS and fm-HS on blood were determined as activated partial thromboplastin time activity (APTT activity) and expressed as IU/mg, as described by the manufacturer (Sigma Chemical Co.)

Proliferation measurement

It has been recently reported (Tzanakakis *et al* 1997) that following a 48-h cell culture the incorporation of [3 H]thymidine as well as the PG synthesis, expressed as incorporated [35 S]-radioactivity, for both cell lines, in the absence of GAGs, was linearly depended with time, retaining a constant rate for first and second day after changing the culture medium

In order to evaluate the time-dependence of the effect of exogenously added GAGs [3 H]-thymidine incorporation, a kinetic study was first performed using 10 μ g/ml of each GAG and incubating the cells in serum-free RPMI-1640 based medium for 24, 48, 72 and 96 h. For practical reasons and to ensure a high thymidine incorporation (Tzanakakis *et al* 1997), in all cases the cells were pulsed with [3 H]thymidine (74 GBq/ml) for 16 h, before the end-point of the experiment. The incorporation of radioactive nucleotide was measured in an LKB beta counter after collecting the cells on a filter paper, using a Titertek Cell Harvester. According to these pilot experiments (see below) a 48-h culture was selected for studying the effect of GAG concentration on cell proliferation.

Statistical analysis

Significant differences between the cell proliferation of the two cell lines in the presence and absence of the various GAGs were evaluated by Student's *t*-test and the two-way completely randomized variance analysis (anova) using the Microcal Origin software (version 3.5).

RESULTS

Purity and chemical composition of GAGs

GAGs, before being added to cell cultures, were analysed for their charge-density and homogeneity of size by electrophoresis in cellulose acetate membranes (Hata & Nagai 1972) and high-resolution HPLC (Engfeldt *et al* 1982, Arvaniti *et al* 1994). As shown in Figure 1, all GAGs tested migrated as homogeneously charged populations, without any contaminating substances being present. Gel-permeation HPLC revealed that all GAGs eluted as homogeneous-sized populations (not shown). The chemical composition of these GAGs is shown in Table 1. Heparin from porcine intestinal mucosa (M_r 12.5 kDa) has a high anticoagulation activity (155 IU/mg). Cleavage of heparin with heparin lyases I, II and III in combination and disaccharide analysis by HPLC (Karamanos *et al* 1997) and HPCE (Karamanos *et al* 1996a) revealed that trisulphated disaccharide Δ di-tri(2,6,N)S (68%) is the commonest disaccharide unit. Most uronic acid ($\approx$ 67%) is IdoA and almost all of glucosamine (GlcN) (87%) is *N*-sulphated. Fast-moving HS from bovine intestinal mucosa showed a composition similar to that of heparin, i.e. Δ di-tri(2,6,N)S was the commonest

Figure 1. Electrophoretic profile of GAGs used for proliferation studies. Solutions of GAGs (2 μ l of 1% (w/v)) were electrophoresed in cellulose acetate membranes in 0.1 M pyridine buffered at pH 3.1 using formic acid, using 0.5 mA per cm of membrane width. GAGs were visualized with toluidine blue. Distilled water was used for destaining.

disaccharide unit (38%) and large contents of IdoA and *N*-sulphated GlcN were present (81% of uronic acid and 74.5%, respectively). This GAG, however, has lower anticoagulation activity (60 IU/mg) and lower relative molecular mass (7.5 kDa) than heparin. HS differs significantly from heparin and fm-HS in both chemical composition and biological properties. The M_r is much higher (48 kDa) and it shows minimal anticoagulation activity (< 0.5 IU/mg). HS mainly contains GlcA (83%) and *N*-acetylated GlcN (61%), whereas according to the HPLC analysis, the main disaccharide unit is Δ di-nonS (35.3%).

Analysis of GalAGs revealed that 93% of uronate in DS is IdoA, whereas CSA and CSC contain only GlcA. GalN was found to be exclusively *N*-acetylated in all types of GalAGs used. HPLC analysis after treatment with chondroitinases showed that the main disaccharide unit in DS and CSA is Δ di-mono4S (71.3% and 68.3%, respectively), whereas in CSC the main disaccharide unit is Δ di-mono6S (63.1%) (Table 1).

Kinetic study on [3 H]thymidine incorporation in the presence of GAGs

In order to evaluate the time-dependence effect of GAGs on [3 H]thymidine incorporation, both cell lines were cultured in the absence and presence of GAGs at the concentration of 10 μ g/ml. As shown in Fig. 2b, CSB acts as a stimulator of [3 H]-thymidine incorporation in STAV-AB, resulted in a time depended increased during days 1–4. CSC, CSA, HS and HA had no significant effects, whereas the maximum recording inhibitory effects of heparin and fm-HS was observed at day 2. A slight suppression of incorporated [3 H]thymidine induced by KS was also recorded at day 2, whereafter no further effect was recorded. Taking into account that the incorporation of radioactive thymidine in the STAV-FCS cell line was

Table 1. Fine chemical composition and properties of GAGs estimated after specific enzymic degradation and HPLC/HPCE analyses and biological assays

Components/properties	Heparin	fm-HS	HS	DS	CSA	CSC
Uronic acid ^a						
IdoA/GlcA	67/33	81/19	17/83	93/7	0/100	0/100
Hexosamines ^b						
N-acetylated	13.0	25.5	61.0	100	100	100
N-sulphated	87.0	74.5	39.0	ND ^d	ND	ND
Disaccharide composition ^c						
Δdi-nonS	0.5	9.0	35.3	2.0	5.0	1.3
Δdi-mono4S	—	—	—	71.3	68.3	13.1
Δdi-monoNS	0.5	5.5	10.0	—	—	—
Δdi-mono6S	6.0	2.5	6.7	1.0	16.5	63.1
Δdi-mono2S	1.5	5.0	8.0	—	—	—
Δdi-di(2,N)S	4.7	14.0	9.8	—	—	—
Δdi-di(2,4)S	—	—	—	24.1	0.5	3.5
Δdi-di(2,6)S	6.5	14.0	11.0	1.6	7.2	14.0
Δdi-di(6,N)S	12.0	12.0	10.5	—	—	—
Δdi-di(4,6)S	—	—	—	—	2.5	5.0
Δdi-tri(2,6,N)S	68.0	38.0	8.7	—	—	—
Properties						
M _r (kDa)	12.5	7.5	48	18.1	16.8	—
APTT (IU/mg)	160	60	< 0.5	—	—	—

Results are expressed as a percentage of ^atotal uronic acid, ^btotal hexosamine and ^ctotal Δ-disaccharides recovered by HPLC and HPCE analyses ^dNot detected

similarly time-dependent by the various GAGs (Fig. 2a), the effect of concentration of various GAGs on cell proliferation was further studied following a 48-h culture

Effects of GAG type and concentration on cell proliferation of human malignant mesothelioma cells

The effect of GAGs on cell proliferation in both cell lines cultured in serum-free RPMI-based medium was evaluated by comparing the proliferation of cells cultured in the absence of GAGs with that in the presence of various GAGs at different concentrations. The results were expressed as a percentage of both [³H]thymidine incorporation and cell count in relation to controls (mean ± SD, *n* = 12). Viability tests showed cell viability exceeding 87%.

In the presence of GalAGs, the fibroblast-like cells responded differently from the epithelially differentiated ones (Fig. 3). STAV-FCS proliferation is stimulated by all GalAGs tested (Fig. 3a–c). DS stimulated cell proliferation in a dose-dependent way even at very low concentrations (0.1 μg/ml) (Fig. 3a) up to a concentration of 10 μg/ml, thereafter no further effect on cell proliferation was recorded (Fig. 3a). CSA and CSC showed stimulatory effects which were pronounced in higher concentrations (50 g/ml) (Fig. 3b,c). In STAV-AB cells, DS was the only GalAGs with a stimulatory effect on cell proliferation (Fig. 3d). CSA and CSC shared no significant effect independently of the concentration used (Fig. 3e,f).

IdoA-containing GlcAGs showed similar effects independently of the cell phenotype. Thus, heparin and fm-HS significantly inhibited the cell proliferation of both cell lines (Fig. 4a,c,d,f), whereas HS did not affect cell proliferation even at very high concentrations (100 μg/ml) (Fig. 4b,e). Heparin showed the strongest suppression of all GAGs tested. Thus,

Figure 2. Kinetic study of the time-dependence effect on [³H]thymidine incorporation of the STAV-FCS (a) and STAV-AB (b) cell lines in the absence and presence of various GAGs at a concentration of 10 g/ml.

Figure 3. Effects of DS (a and d), CSA (b and e) and CSC (c and f) on [^3H]thymidine incorporation and cell number of STAV-FCS (a, b and c) and STAV-AB (d, e and f) cells. Results are presented as percentage [^3H]thymidine incorporation (■) and cell number (○) with respect to control. The results are the average $\pm$ SD of three experiments in triplicate. Asterisks (*) show the significant differences at a level $P \leq 0.05$ and (+) at the level $P \leq 0.01$.

even at a very low concentration of 0.1 $\mu\text{g/ml}$ heparin resulted in $\approx 50\%$ inhibition, and an inhibition of 85–90% was obtained at 10 $\mu\text{g/ml}$, whereafter an almost complete inhibition was found (Fig. 4a,d). Fm-HS also inhibited cell proliferation (Fig. 4c,f), but the highest inhibition percentages obtained using 100 $\mu\text{g/ml}$ were $\approx 70\%$ and 30% for STAV-FCS and STAV-AB, respectively. The different functions of HS on both cell lines as compared to heparin and fm-HS may well be attributed to the differences found in their chemical composition and structure (Table 1).

HA showed a 25% inhibitory effect at the highest concentration tested (100 $\mu\text{g/ml}$) only for STAV-FCS (Fig. 5a), whereas the proliferation of STAV-AB cells was not affected (Fig. 5c). KS had an inhibitory effect on both cell lines, but this was more pronounced on the STAV-AB (30%) than on the STAV-FCS cell line (10%) at the highest concentration of 100 $\mu\text{g/ml}$.

DISCUSSION

Human malignant mesothelioma cells of both phenotypes produce HA, GalAGs and HS. The latter GAGs are localized at both the cell membrane and extracellular medium. The amounts of these GAGs and their distribution between the cell membrane and culture medium depend on the cell phenotype (Tzanakakis *et al.* 1995a).

GAGs are highly heterogeneous macromolecules, their heterogeneity being derived from the disaccharide unit that constitutes the primary structure. Differences are found in the types of uronic acid and hexosamine, in the number and position of sulphate residues, in the presence of *N*-acetyl and/or *N*-sulphate groups and in the relative molecular mass. The variations in primary structure produce molecules with various chemical and biological properties. It is known that GAGs participate in the regulation of several cellular events, including cell proliferation, and their structural variability must be considered when studying such functions.

It has been reported that heparin exerts a significant antiproliferative effect on several cells (Wright *et al.* 1989a, Volpi *et al.* 1993). These studies revealed that the high contents of IdoA and *N*-sulphated GlcN, are all essential factors in GAGs, such as heparin and fm-HS, that affect their ability to inhibit cell proliferation. Considering the inhibitory effects by heparin and fm-HS that we demonstrated for STAV-FCS and STAV-AB, one should note that the presence of high amounts of IdoA-derived trisulphated disaccharides, i.e. $[\text{IdoA}(2\text{-O-SO}_3^-)\text{-GlcNHSO}_3^-(6\text{-O-SO}_3^-)]$, responsible for the overall negative charge of these GlcAGs, should be considered a critical factor for the inhibition of cell proliferation (Wright *et al.* 1989a, Castellot *et al.* 1985b, Petitou *et al.* 1991). Although HS is composed of the same core-repeating disaccharide unit as heparin and fm-HS, this GlcAG differs in sulphation pattern and the anticlotting activity when compared with those of heparin and fm-HS. Moreover, this GAG caused no significant effects on proliferation in any of the cell lines we studied. This is a strong indication that the fine chemical structure of the repeating disaccharide unit in respect to sulphation degree and distribution is a key factor determining these effects of the polysaccharide. Therefore, chain geometry, which is dependent on IdoA content (Scott 1994) and overall charge density, which depends in turn on the sulphate residues present (Karamanos & Hjerpe 1996), is a parameter of great importance in this context.

GalAGs, i.e. DS, CSA and CSC, are strong stimulators of STAV-FCS proliferation, whereas only DS had stimulatory effect on STAV-AB, showing that the presence of IdoA or GlcA is a very important factor, where the presence of flexible IdoA residues provides various conformations to these macromolecules. Therefore, IdoA-containing chains (DS) had only

Figure 4. Effects of heparin (a and d), HS (b and e) and fast-moving HS (c and f) on $[^3\text{H}]$ thymidine incorporation and cell number of fibroblast-like (a, b and c) and epithelially differentiated (d, e and f) cells. For expression of results see Figure 2.

Figure 5. Effects of HA (a and c) and KS (b and d) on $[^3\text{H}]$ thymidine incorporation and cell number of fibroblast-like (a and b) and epithelial differentiated (c and d) cells. For expression of results see Figure 2.

stimulatory effect, whereas the effect of GlcA-containing sulphated chains (CSC or CSA) is depending on the cell phenotype. The sulphation of the chain is also of major importance. Relating the biological activities of macromolecules to their type and sulphation one finds that a key structural factor in an increasing proliferation activity is the presence of the disaccharide unit [$\text{IdoA-GalNAc-(4-O-SO}_3^-)$], which is the major component of DS chain (71.3%).

The type of hexosamine present, e.g. GlcN or GalN, as well as the anomeric conformation of the glycosidic bond (α or β) in the GAG repeating unit, seems very important, since only GlcAGs can be inhibitors of both STAV-AB and STAV-FCS cell proliferation.

The exogenous addition of HA in cultures showed a slight inhibitory effect, which is seen only for fibroblast-like mesothelioma cells (25% at 100 µg/ml), whereas this polysaccharide makes no significant contribution to the regulation of STAV-AB cell proliferation. This is

worth noting since HA is the principal GAG produced by STAV-AB cells (6–8 times larger amount than STAV-FCS) (Tzanakakis *et al* 1995a) and this polysaccharide in many cases is produced in excessive amounts by native mesotheliomas (Nurminen *et al* 1994, Karamanos & Hjerpe 1997). KS is also a component of some connective tissue PGs. The slight inhibitory effect of this GAG may indicate yet another interaction between the tumour cells and the tumour stroma. This finding would suggest that mesothelioma cells retain interactions with the surrounding matrix, although it has been proposed that such interactions have been diminished in other transformed cells (Westergren-Thorsson *et al* 1993, Heldin & Westermark 1989).

The molecular size of the polysaccharide chain does not seem to be an essential factor for regulating cell proliferation as DS and CS of approximately similar M_r (18.1 and 16.8 kDa, respectively) had different effects on mesothelioma cell proliferation. Although binding of GAGs to proteins is not usually permitted when critical M_r (48 kDa) values are not exceeded (Jackson *et al* 1991), HS of higher M_r than those of heparin (12.5 kDa) and fm-HS (7.5 kDa) caused no significant decrease in cell proliferation.

It is known that GAG chains form complexes with growth factors and regulate their activity by protecting them from proteolytic cleavage or by presenting them to their receptors (Saksela *et al* 1988). It should be noted that the participation of growth factors in cell proliferation by the formation of complexes with GAGs requires their binding to specific GAG structures after involving the length of a few disaccharides. Mesothelioma cells produce an extracellular matrix rich in GAG chains. Changes in the composition of this extracellular matrix are therefore able to affect the fine regulation of proliferation or even influence the cell phenotype. The precise mechanism by which the GAGs influence the mesothelioma to synthesize DNA in S-phase is still greatly unknown. It is not yet clear exactly how the cell cycle is altered or if the effects on proliferation depend on the interactions with specific growth factors or receptors. Furthermore, the fine structural background to these effects of GAGs remain to be elucidated. Further studies to elucidate different mechanisms of GAGs as regulators of mesothelioma and mesothelial cell proliferation are in progress in our laboratories.

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