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Modifications of proteoglycans secreted into the growth medium by young and senescent human skin fibroblasts

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Abstract The properties of proteoglycans (PGs) secreted into the growth medium by normal young and senescent human skin fibroblasts (HFs) were investigated. In both cases, the incorporation per cell of radioactive precursors into total PGs was similar. The polysaccharide chains of PGs from young and senescent HFs were mainly represented by galactosaminoglycuronans and showed a similar range of size distribution. However, galactosaminoglycuronans of PGs secreted by senescent HFs had a lower content of unsulphated disaccharides and a lower proportion of D-glucuronosyl residues. Moreover, senescent HFs released into the growth medium higher relative amounts of small PGs with chondroitin sulphate, dermatan sulphate chains, such as decorin.

Key words: Proteoglycan; Glycosaminoglycan; Fibroblast; Growth medium; Replicative senescence

1. Introduction

Normal human fibroblasts (HFs) in culture have a limited ability to proliferate [1]. This phenomenon has been termed 'cellular senescence' and a number of observations support the view that it reflects some aspects of aging in vivo [2–4]. In addition to the loss of proliferative capacity, the phenotype of senescent HFs displays various changes, including an increase in cell size, a consequent decrease of cell number at confluence and alterations in subcellular structure [2,3]. Senescence also involves modifications of cell metabolism. Quantitative and qualitative changes in the synthesis of certain proteins [5–8] suggest that both efficiency and specificity of the biochemical machinery for protein synthesis may be different in senescent cells. These changes also affect the main components of extracellular matrix (ECM), such as fibronectin [9,10] and collagen [5,11]. We have found that the biosynthesis of proteoglycans (PG) is also modified with senescence [12], since late passage HFs incorporate greater amounts of radioactivity in the large PGs of the cell layer and show a lower ratio between PGs containing dermatan sulphate (DS) and those containing heparan sulphate (HS) [12].

In this paper we investigated the structural properties of the PGs secreted into the growth medium by early and late passage HFs. It appears that senescent HFs secrete PGs containing galactosaminoglycuronan chains with a greater level of both sulphate and iduronate residues and release higher amounts of small PGs containing chondroitin sulphate (CS) and DS, such as decorin.

2. Materials and methods

[^{35}S]Sulphate (51.2 mCi mmol^{-1}), [^{3}H]leucine (73 mCi mmol^{-1}), [^{3}H]glucosamine (28 mCi mmol^{-1}), [^{14}C]leucine (311 mCi mmol^{-1}) and the scintillator enhancer Amplify were obtained from Amersham (Little Chalfont, UK).

Seikagaku (Tokyo, Japan) provided the following C-O lyases: chondroitinase (Chase) ABC from *Proteus vulgaris*, Chase ACII from *Arthrobacter aurescens*, heparinase and heparitinase from *Flavobacterium heparinum*. Papain (type III from papaya latex) was from Sigma (St. Louis, MO).

Sigma (St Louis, MO) supplied the standard disaccharides: [α-ΔUA-[1→3]-GalNAc (Δdi-0S); α-ΔUA-[1→3]-GalNAc-4S (Δdi-4S); α-ΔUA-[1→3]-GalNAc-6S (Δdi-6S); α-ΔUA-2S-[1→3]-GalNAc-4S (Δdi-2,4S)].

HF cultures were established, grown in monolayers and subcultured as previously described [12]. The proliferative capacity of HFs declined progressively with increasing passages. HFs at early (4–5) and late (28–31) passages, i.e. young and senescent HFs, were used for the experiments. Total DNA content in the flasks was measured using Hoechst 33258 [13].

Cultures of young and senescent HFs were labelled at early confluence with either [^{3}H]glucosamine (30 μCi ml^{-1}) or [^{35}S]sulphate (20–30 μCi ml^{-1}) to study the polysaccharidic component of PGs. In the experiments for core protein study, cells were labelled with either [^{3}H]leucine (20–30 μCi ml^{-1}) or [^{14}C]leucine (15 μCi ml^{-1}). The cells were left to incorporate the radioactive isotopes for 48 h at 37 C° under a humidified atmosphere.

The growth medium was quantitatively recovered in saline PBS, supplemented with protease inhibitors (10 mM EDTA-disodium salt, 5 mM benzamidine hydrochloride, 100 mM ε-aminocaproic acid, 1 mM phenylmethylsulphonyl fluoride, 10 mM *N*-ethylmaleimide). Macromolecular material was collected by gel filtration on Bio-Gel P2 column (30×4 cm), equilibrated and eluted with 50 mM Na acetate, pH 6.0, containing 150 mM NaCl, 8 M urea, 0.5% (v/v) Triton X-100 and protease inhibitors (buffer A). A crude PG fraction was then isolated from proteins and hyaluronic acid by chromatography on DEAE-Sephacel column, (10×1 cm) equilibrated with buffer A and eluted with a continuous NaCl gradient, ranging from 0.15 to 1.2 M, in buffer A.

The collected samples were frozen until use, after having exchanged buffer A with 50 mM sodium acetate, pH 6.0, containing 4 M guanidine hydrochloride and protease inhibitors (buffer B) [12].

Information about GAG composition was obtained by enzymatic digestion of PGs with specific C-O lyases: Chase ABC and Chase ACII [12], heparinase and heparitinase [14]. The sulphation pattern of galactosaminoglycuronan chains was analysed by HPLC fractionation of the unsaturated disaccharides released by Chase ABC and Chase ACII [15], using 5 nmol of standard disaccharides as cold carrier.

GAG chains were then freed of protein by papain treatment (1U 40 000 dpm^{-1}) [16] and protease-resistant peptides were removed from GAGs by β-elimination [17]. GAG chains were analysed in a FPLC apparatus by ion exchange chromatography on a Resource column (Pharmacia) (column volume 1 ml, flow rate 2 ml min^{-1}), equilibrated in water and eluted with a linear gradient of NaCl, ranging from 0 to 2 M in 100 ml and by gel filtration chromatography on a Sephacryl S-300 column, (90×1 cm) equilibrated and eluted with 0.5 M NaCl.

The sulphation pattern of the different galactosaminoglycuronans was determined by HPLC as reported above. The sensitivity and the resistance of the chains to the enzyme treatments were also tested by gel-filtration chromatography on Bio-Gel P6 column (120×1 cm),

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equilibrated and eluted with 0.5 M NH_4HCO_3, performed before and after enzyme digestion.

Crude PG preparation was partially purified by hydrophobic interaction chromatography on Octyl-Sepharose column (6.4×1 cm), equilibrated with buffer B and eluted with a linear gradient of Triton X-100, ranging from 0 to 0.8% (v/v), in buffer B.

The radioactive material recovered from Octyl-Sepharose was analysed by gel-filtration chromatography on Sepharose CL-4B, (50×1 cm), equilibrated and eluted with buffer B.

SDS-polyacrylamide gel electrophoresis (PAGE) analysis [18] (10% gel) was used to study [^{14}C]leucine-labelled hydrophobic PGs, before and after treatment with Chase ABC, heparinase and heparitinase, yielding free core proteins. Radioactive bands were then detected by autoradiography, using Kodak X-Omat AR films with an exposure of 30–40 days at −80C°. Densitometric analysis of the autoradiographs was performed with a laser densitometer Ultroscan (LKB). The identification of small PG core proteins was performed according to Fisher et al. [19] after blotting on nitrocellulose, using specific rabbit antisera (1:200) against human recombinant decorin (LF122) and human biglycan synthetic peptide (LF 51), both prepared and kindly provided by Dr. L.W. Fisher, NIH, Bethesda, MA.

The hexuronate pattern of hydrophobic PGs was studied by mapping the oligosaccharides produced from galactosaminoglycuronans by Chase ACII digestion. Gradient PAGE was used to resolve the various oligosaccharide fractions on the basis of their size [20]. After electrophoresis the radioactive material was blotted on a positively charged nylon membrane (Pall Europe, Portsmouth, UK) by a Trans-Blot SD semi-dry transfer cell (BioRad, Hercules, CA). The membrane was then sprayed with the scintillation enhancer Amplify and the radioactive bands of oligosaccharides were detected by autoradiography and analysed by a laser densitometer as reported before.

Radioactivity was measured with a Packard 2300 TR liquid scintillator counter. The Emulsifier Scintillator Plus (Packard) was used as scintillator cocktail.

3. Results

Senescent HFs had a larger size than young HFs. Under our experimental conditions, a typical plastic flask of 75 cm^2 contained a monolayer consisting on average of $4.6 \pm 0.3 \times 10^6$ young HFs and $2.8 \pm 0.3 \times 10^6$ senescent HFs. The total DNA content in the flask was 22.0 ± 1.0 and 13.4 ± 0.4 μg, respectively and the calculated ratio DNA/cell (expressed as pg) amounted to 4.8 in both cases, indicating that young and senescent HFs were in about the same phase of the cell cycle. Considering the different cell number per flask, young and senescent HFs incorporated per cell a very similar average amount of radioactive precursors into GAG chains and core proteins of isolated PGs.

Table 1
Pattern of disaccharides (expressed as percentage) in galactosaminoglycuronan chains of secreted proteoglycans

Sample	HFs	Δdi-0S	Δdi-4S	Δdi-6S	Δdi-2,4S
Crude PGs	young	74.7	22.1	2.5	0.7
	senescent	49.1	41.1	3.1	6.6
GAGs					
Fraction b	young	100	n.d.	n.d.	n.d.
	senescent	97.1	2.9	n.d.	n.d.
Fraction d	young	2.9	85.8	6.8	4.4
	senescent	3.0	80.9	8.8	7.3

Cells were labelled with [^{3}H]glucosamine. n.d., non-detectable. See Section 2 and text for details about the isolation of crude PGs, the separation of their GAG chains freed of proteins and the analysis of enzymatically released unsaturated disaccharides. Fractions b and d are referred to the separation reported in Fig. 1.

In all the experiments with young and senescent HFs, the crude PG fraction emerged from DEAE-Sephacel column as a single peak, at NaCl concentrations ranging from 0.6 to 0.7 M. About 90% of GAGs contained in these PGs underwent complete degradation by combined digestion with Chase ABC and Chase ACII and were thus identified as galactosaminoglycuronans. Their sulphation pattern demonstrated that PGs secreted by young HFs contained a greater amount of unsulphated disaccharides (Table 1).

GAG chains freed of protein by papain digestion and β-elimination were resolved by Resource column chromatography into 4 different fractions (Fig. 1). The relative ratio of fraction b to fraction d decreased with cellular senescence, amounting to 0.8 and 0.3 in young and in senescent HFs, respectively. Fractions a, b, and d did not contain significant amounts of HS, as they resisted heparinase-heparitinase digestion. Fractions a and d were fully degraded by Chase ABC treatment and were identified as CS, DS chains. In contrast, fraction b required combined treatment of Chase ABC plus Chase ACII for complete degradation and this could well be related to a very low degree of sulphation [21]. For fractions b and d, containing sufficient radioactive material for the analysis, the GAG chain composition was confirmed by HPLC analysis of the enzymatically released, unsaturated disaccharides. As reported in Table 1, fractions b and d consisted

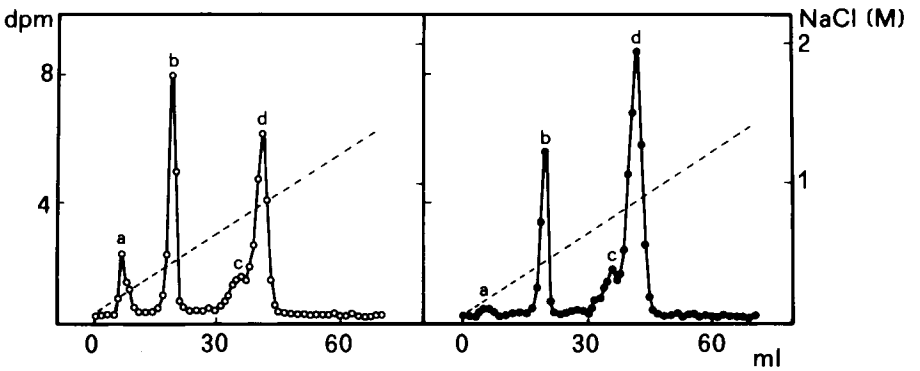

Fig. 1. Ion-exchange chromatography on Resource column of glycosaminoglycans obtained from crude growth medium proteoglycans. (○—○) Young HF; (●—●) senescent HF; (- - -) NaCl (M). Determination of dpm is expressed in thousands. Cells were labelled with [^{3}H]glucosamine. GAGs were freed of protein core by papain digestion of crude PGs. Protease-resistant peptides were removed from GAGs by β-elimination. The percentage relative contents of the different peaks were as follows: young HF: peak a 10.6, peak b 34.4, peak c 14.3, peak d 40.7; senescent HF: peak a 1.9, peak b 21.6, peak c 14.2, peak d 62.3.

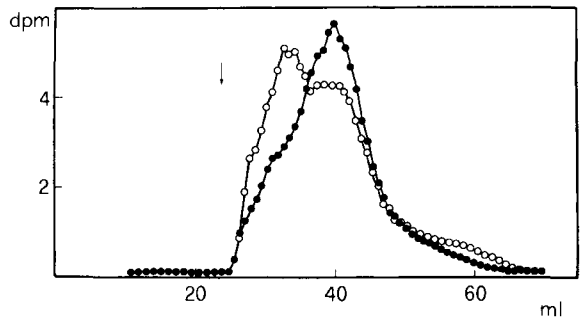

Fig. 2. Gel filtration chromatography on Sephacryl S-300 of glycosaminoglycans obtained from crude growth medium proteoglycans. (○—○) Young HF; (●—●) senescent HF. Cells were labelled with [^{3}H]glucosamine. Determination of dpm is expressed in thousands. (↓) Void volume. GAGs were freed of protein core by papain digestion of crude PGs isolated from the growth medium. Protease-resistant peptides were removed from GAGs by β-elimination.

mainly of unsulphated (very likely chondroitin) and 4-sulphated galactosaminoglycuronan chains (CS and DS), respectively (Table 1). Fraction c was identified as HS, since it resisted Chase ABC digestion and was degraded by heparinase-heparitinase treatment.

The chain size of radiolabelled GAGs secreted by young and senescent HFs was quite polydispersed within the same range of values, although large size GAGs prevailed in young HFs (Fig. 2).

In both young and senescent HFs, chromatography of the crude PGs on Octyl-Sepharose CL-4B yielded a partially purified PG fraction, which emerged from the column at the Triton X-100 concentration from 0.22 to 0.4% and comprised about 70% of the loaded radioactivity. This hydrophobic PG fraction contained about 90% of galactosaminoglycuronan chains, as indicated by the sensitivity to Chase ABC and Chase ACII treatment.

Gel filtration of the hydrophobic PG fraction on Sepharose CL-4B under dissociative conditions [12] yielded a radioactive profile with two evident peaks (K_{AV} 0.08 and 0.40, respectively) (Fig. 3A). Very likely they consisted mainly of large CS,DS-PGs and small CS,DS-PGs, corresponding to versican and decorin-biglycan, respectively [22]. The relative contribution of small PGs to the total ^{35}S radioactivity increased markedly in senescent HFs, amounting to 41% in young HFs and 61% in senescent HFs (Fig. 3A). This finding was also confirmed by the analysis of isolated hydrophobic PGs, labelled with [^{14}C]leucine, of young and senescent HFs on SDS-PAGE, performed before and after digestion with specific C-O lyases. In undigested samples, containing the same amount of radioactive material (100 000 dpm), the protein band of about 115 kDa was markedly more evident in senescent HFs. According to densitometric analysis, this band amounted to 30.6 and 51.7% of the total radioactivity in young and senescent HFs, respectively (Fig. 3B, lanes a,d). After treatment with C-O lyases, the 115 kDa band disappeared, being apparently replaced by a band with an approximate molecular mass of 46 kDa (Fig. 3B, lanes b,e). When treated with specific antisera, this band showed a positive reaction as decorin core protein with a typical doublet and a negative response as biglycan core protein (Fig. 3B, lanes c,f) [23–25].

Oligosaccharide mapping, performed by gradient PAGE after cleavage of the β 1→4 linkage to glucuronosyl residues by Chase ACII, showed that hydrophobic PGs secreted by senescent HFs had an increased amount of oligosaccharidic chains with relatively high degree of polymerization (Fig. 4). This is consistent with an increased content of L-iduronosyl residues, whose linkage resists Chase ACII cleavage [20].

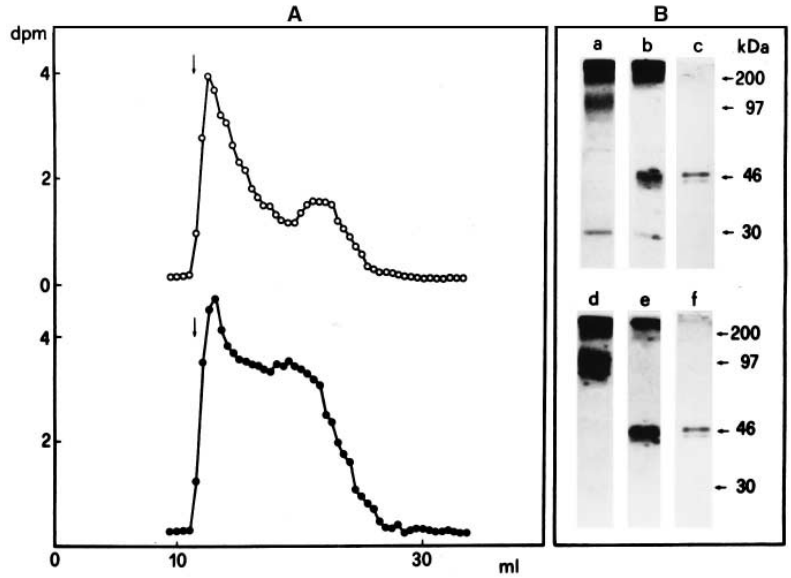

Fig. 3. (A) Gel filtration chromatography on Sepharose CL-4B of hydrophobic proteoglycan fraction. (○—○) Young HFs; (●—●) senescent HFs. Cells were labelled with [^{35}S]sulphate. Determination of dpm is expressed in hundreds. (↓) Void volume. (B) SDS-PAGE of hydrophobic proteoglycan fraction. Undigested PGs (young HFs, a; senescent HFs, d); PG core proteins recovered after treatment with Chase ABC, heparinase and heparitinase (young HFs, b; senescent HFs, e). Core proteins tested with antiserum raised against decorin and a peroxidase-conjugated second antiserum (young HFs, c; senecescent HFs, f). Cells were labelled with [^{14}C]leucine. (a,b,d,e) Detection by autoradiography; (c,f) detection by immunoblotting. For experimental details see text.

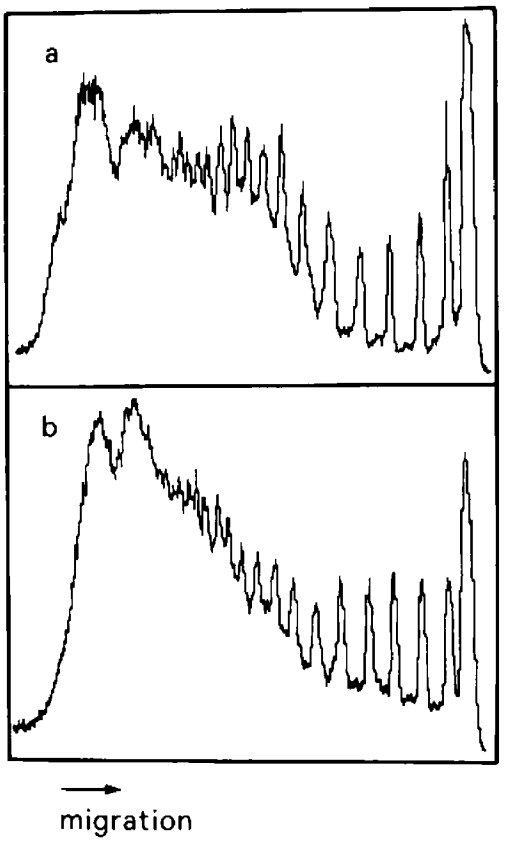

Fig. 4. Polyacrylamide gel electrophoresis of chondroitinase ACII digest of hydrophobic proteoglycan fraction. (a) Young HFs; (b) senescent HFs. Cells were labelled with [^{35}S]sulphate. For experimental details see the text.

4. Discussion

The incorporation per cell of radiolabelled precursors into crude PGs from the growth medium did not differ significantly in young and senescent HFs. This suggests that the biosynthesis of this PG fraction did not decrease with replicative senescence and is consistent with the previous finding that senescent HFs maintain a normal energy charge [12] and much of their anabolic capacities [2].

The chromatographic properties of PGs suggest that the total polyanion charge was maintained in late passage cells, as well as the size distribution of GAG chains, which were mainly represented by galactosaminoglycuronans. However, PGs secreted by senescent HF contained galactosaminoglycuronans with a higher sulphation degree and a lower percent content of totally unsulphated chains. The decrease of unsulphated periods in galactosaminoglycuronans is also a typical age-dependent change of PG chemical structure in vivo [26].

It is noteworthy that the percentage decrease of unsulphated disaccharides was roughly equivalent to the percentage increase of 4-sulphated disaccharides. Therefore, cellular senescence is likely to affect the enzyme activities (sulphotransferase and/or sulphatase) responsible for the presence of sulphate residues on C4 of galactosaminyl residues.

Galactosaminoglycan chains from PGs secreted by senescent HFs contained a lower proportion of D-glucuronosyl residues. This result is linked with the concomitant increase in 4-sulphation and agrees well with the established fact that epimerization of D-glucuronosyl residues to L-iduronosyl residues is followed by 4-sulphation of adjacent *N*-acetylgalactosaminyl residues [23]. The shift of glucuronosyl to iduronosyl residues in the polysaccharides is of importance for their secondary structure, since the former residues exist predominantly in only one conformation, whereas the latter ones show an equilibrium of three different conformers [27]. As a consequence, the modified copolymeric structure of CS,DS chains might affect their physical interactions with other molecules.

The relative proportions of different PG populations secreted into the growth medium changed with replicative senescence, as the relative content of small CS,DS-PGs increased significantly in senescent HFs. Immunological identification showed that they consisted mainly of decorin. The increased secretion of decorin into the growth medium by late passage HF cultures might contribute to the decline of proliferative capacity. In fact, decorin core protein was found to bind transforming growth factor β [28,29], thus inactivating the cytokine [28]. Moreover, the overexpression of decorin in Chinese hamster ovary cells was shown to inhibit cell proliferation [30].

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