Modifications of proteoglycans extracted from monolayer cultures of young and senescent human skin fibroblasts

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Abstract Proteoglycans (PGs) were extracted from culture monolayers of human skin fibroblasts (HFs) at early and late passages. Total PGs from senescent cells had markedly reduced abilities to bind type I collagen and hyaluronic acid, but retained normal binding properties with fibronectin and laminin. The constituent polysaccharides of PGs were comparatively characterised. PGs recovered from young and senescent HF cultures had equivalent total polyanionic charges and similar size distributions of the glycosaminoglycan chains. This applied to both types of polysaccharide chains found in PGs, namely the galactosaminoglycuronans (GalN-GAGs) and the glucosaminoglycuronans (GlcN-GAGs). However, senescent HFs produced a greater proportion of PGs containing GlcN-GAG chains and increased the sulphation of the remainding PG fraction with GalN-GAG moieties, yielding a major gain of C6-sulphate groups in the galactosamine residues.

Key words: Proteoglycan; Glycosaminoglycan; Fibroblast; Culture monolayer; Replicative senescence

1. Introduction

The term 'cellular senescence' is currently used to identify the progressive decline in proliferative capacity that cells of higher eukaryotes undergo in culture, when they are allowed to propagate in a continuous series of population doublings. This phenomenon occurs in normal cell cultures from many animal species, but it is particularly stringent in human cells [1]. The relationship between senescence in culture and aging in vivo is still tenuous; however, several observations support the view that cellular senescence is a manifestation of processes that occur during aging in vivo [1–5].

In addition to the loss of proliferative capacity, senescent cells display a number of phenotypic changes, including an increase of cell size [5] and variations of extracellular matrix (ECM) components [6–9]. With reference to proteoglycans (PGs), senescent human skin fibroblasts (HFs) release into the growth medium an increased proportion of decorin and secrete PGs containing galactosaminoglycuronan (GalN-GAG) moieties with a higher sulphation degree of galactosamine residues and a lower percentage content of totally unsulphated polysaccharide chains [10]. Otherwise, senescence-dependent changes of PGs organised in the cell membranes and in the ECM of HF monolayers are poorly known.

This paper reports a comparative study of the PGs extracted from HF monolayer cultures at early and late passages. It appeared that total PGs from senescent HFs had markedly reduced abilities to bind collagen type I and hyaluronic acid and comprised a greater proportion of PGs with glucosaminoglycuronan (GlcN-GAG) chains. Moreover, the PG fraction with GalN-GAG chains from senescent cells had a net increase of C6-sulphation in the galactosamine residues.

2. Materials and methods

2.1. Materials

[^{3}H]Glucosamine (28 mCi/mmol) was purchased from Amersham (Little Chalfont, UK). Seikagaku (Tokyo, Japan) supplied the depolymerising C-O lyases: chondroitinase (Chase) ABC from *Proteus vulgaris*, Chase ACII from *Arthrobacter aurescens*, heparinase and heparitinase from *Flavobacterium heparinum*. Collagen type I from human skin and fibronectin from human plasma were generous gifts of Dr R. Tenni and Dr P. Speziale, respectively (University of Pavia). Sigma (St. Louis, MO) supplied papain (type III from papaya latex), hyaluronic acid from human umbilical cord, laminin from EHS mouse sarcoma and the following 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); α-ΔUA-[1→4]-GlcNAc (Δdi-0S_{HS}); α-ΔUA-[1→4]-GlcNS (Δdi-NS_{HS}); α-ΔUA-[1→4]-GlcNAc-6S (Δdi-6S_{HS}); α-ΔUA-[1→4]-GlcNS-6S (Δdi-6,NS_{HS}); α-ΔUA-2S-[1→4]-GlcNS (Δdi-2,NS_{HS}); α-ΔUA-2S-[1→4]-GlcNS-6S (Δdi-2,6,NS_{HS}). Bovine serum albumin was purchased from Calbiochem (La Jolla, CA). The other biochemicals and reagents were commercial products of the highest purity.

2.2. Culture and ^{3}H-labelling of cells

HF cultures were propagated in continuous subcultures as monolayers according to the technique previously described [11] and cells at early (4–5) and late (28–31) passages, i.e. young and senescent HFs, were used in the study. Total DNA content in the flasks was measured by the Hoechst 33258 dye method [12].

At early confluence the selected early- and late-passage cultures were incubated with [^{3}H]glucosamine (30 μCi/ml) and allowed to incorporate the radioactive compound for 48 h at 37°C under a humidified atmosphere.

2.3. Isolation of ^{3}H-labelled proteoglycans

After ^{3}H-labelling, the growth medium was quantitatively removed and the cell monolayers were carefully washed with saline PBS containing a cocktail of protease inhibitors [10]. The protease inhibitors were included in all buffer solutions used for the various preparative biochemical fractionations. PGs were extracted from HF monolayers for 24 h at 2–4 C° with 4 M guanidine hydrochloride (GuHCl) in 50 mM sodium acetate buffer, pH 6.0, containing 2% (v/v) Triton X-100. Non-incorporated [^{3}H]glucosamine and small-size compounds were removed by gel filtration on Bio-Gel P2 columns and then ^{3}H-labelled PGs were isolated from the extracts by anion-exchange chromatography on DEAE-Sephacel columns eluted with a linear NaCl gradient [11] and stored frozen until use. In all the experiments with young and senescent HFs, the total PG fraction emerged from the columns as a single peak, at NaCl concentrations ranging from 0.6 to 0.7 M, thus exhibiting the same gross polyanion charge.

2.4. Binding assays

The potential binding properties of PGs from young and senescent HFs with different macromolecular components of ECM were assayed

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by a solid-phase binding method [13]. For this purpose large aliquots of total PGs were radioiodinated for 10 min in PBS with two iodo beads (Pierce, UK) pretreated for 5 min with 17 MBq ^{125}I (NEN Dupont, USA), strictly following the manufacturer's instructions. The uncoupled radioactive isotope was removed by chromatography on PD 10 columns (Pharmacia, Sweden) eluted with PBS. ^{125}I-Labelled PGs were tested as specific ligand interacting with the macromolecular substrates immobilised on the wells of microtitre plates. In all assays the bound radioactivity was corrected for the background values of the ^{125}I-labelled material fixed in control wells coated with albumin as aspecific substrate.

2.5. Methods of ^{3}H-labelled proteoglycan characterisation

The different GAG moieties of total ^{3}H-labelled PGs were broken down to their repeating disaccharide subunits with the aid of specific glycan C-O lyases. Chase ABC and ACII depolymerised the GalN-GAG chains of chondroitins and dermatans [14], whereas heparinase and heparitinase degraded the GlcN-GAG chains of heparans [15]. The ^{3}H-labelled unsaturated disaccharides released as terminal products of the enzymatic digestions were then resolved and identified in distinct HPLC analyses performed on a Beckman System Gold, comprising a Supelcosil LC-SAX 250×4.6 mm column protected with a Supelguard LC-SAX 20×4.6 mm column (Supelco Inc., Bellefonte, PA). Unsaturated galactosamine-containing disaccharides were separated as previously described [16]. Unsaturated glucosamine-containing disaccharides were resolved by using a two-step elution with NaH_2PO_4 gradient solution at pH 3.2 (14 min gradient from 5 to 16 mM, followed by 36 min gradient from 16 to 40 mM).

Aliquots of ^{3}H-labelled PGs from young and senescent HF cultures were digested with papain and treated for β-elimination of protease-resistant peptides to obtain GAG chains totally devoid of core protein [10]. Fast liquid chromatography on a Resource anion exchanger column was used to fractionate the protein-free GAGs [10]. The relative proportions of the different types of GAG chains were determined by comparing the elution profiles of runs carried out before and after treatment of the samples with Chase ABC and ACII and with heparinase and heparitinase. The sulphation pattern of separated GAGs was also analysed by the above HPLC distinct fractionations of the unsaturated disaccharides released by the specific C-O lyases. The size distribution of protein-free GAG chains was investigated by gel-filtration chromatography on a Sephacryl S-300 column [10], before and after treatment with Chase ABC and ACII.

2.6. Radioactivity measurements

The ^{3}H-labelled compounds were quantitated in a Packard 2300 TR liquid scintillation spectrometer using the scintillator enhancer Amplify as phosphor cocktail. A Packard Cobra II γ-counter served to monitor ^{125}I-radioiodinated materials.

3. Results

3.1. [^{3}H]Glucosamine incorporation into proteoglycans

Confluent monolayers of HFs at early and late passages comprised a different number of cells. As noted in a previous report [10], senescent HFs yielded 60.9% smaller counts and had 2/3 average larger size than young HFs. However, they comprised the same amount of DNA (4.8 pg/cell), thus implying that the compared cultures contained cells in about the same phase of the cell cycle. When referred to the DNA content and thus corrected for the monolayer cell density, the incorporation of [^{3}H]glucosamine into GAG chains of PGs was similar in the cultures of young and senescent HFs, i.e. $30 \pm 0.5 \times 10^{3}$ and $27 \pm 2 \times 10^{3}$ cpm/μg DNA, respectively.

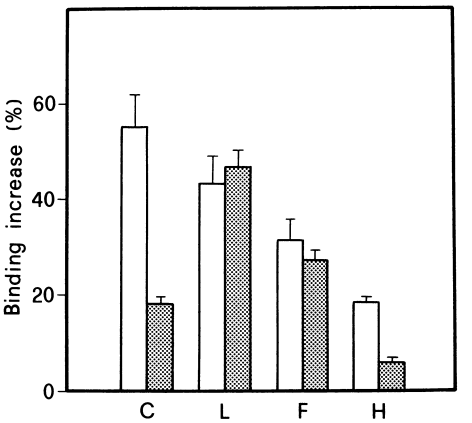

Fig. 1. Solid-phase binding assay of total proteoglycans extracted from culture monolayers of young (open bars) and senescent (hatched bars) human skin fibroblasts with extracellular matrix components. C: collagen type I; L: laminin; F: fibronectin; H: hyaluronic acid. ^{125}I-Labelled total PGs were used for this assay. Data are expressed as per cent increases of [^{125}I]PG binding with specific ligands as compared to the basic aspecific binding with albumin.

3.2. Macromolecular interactions of proteoglycans

The binding properties of total ^{125}I-labelled PGs for different ECM components were altered by cellular senescence. In particular, the bindings to type I collagen and hyaluronic acid were greatly reduced in late-passage cultures, whereas the ones to laminin and fibronectin did not significantly change (Fig. 1).

3.3. Characterisation of ^{3}H-labelled proteoglycans

Substantial portions of GAG chains from total PGs (about 40% and 30% in young and senescent HFs, respectively) were completely depolymerised by Chase ABC and ACII and thus were identified as GalN-GAGs (chondroitinsulphates and dermatansulphates). The Chase ABC- and ACII-resistant GAGs were degraded by combined actions of heparinase and heparitinase and, therefore, consisted of GlcN-GAGs (heparansul-

Table 1
Pattern of disaccharides (expressed as percentage) in galactosaminoglycuronan (A) and glucosaminoglycuronan (B) chains of proteoglycans extracted from culture monolayers of normal human skin fibroblasts

HFs	Δdi-0S	Δdi-4S	Δdi-6S	Δdi-2,4S		
A						
Young	57.9	36.6	4.5	1.0		
Senescent	48.4	30.6	17.0	2.1		
HFs	Δdi-0S_{HS}	Δdi-NS_{HS}	Δdi-6S_{HS}	Δdi-N,6S_{HS}	Δdi-N,2S_{HS}	Δdi-N,2,6S_{HS}
B						
Young	53.8	19.2	9.0	5.8	5.3	6.8
Senescent	51.6	23.1	8.3	5.4	4.8	6.5

^{3}H-Labelled unsaturated disaccharides were released from GalN-GAGs and from GlcN-GAGs by digestion with Chase ABC and ACII and with heparinase and heparitinase, respectively. The released disaccharides were separated and mapped by HPLC as reported in Section 2, using 5 nmol of standard carrier disaccharides.

phates). The analysis of the disaccharide digestion products revealed that the GalN-GAGs synthesised by senescent HFs contained a lower amount of unsulphated disaccharides and had a net increase of C6-sulphated galactosamine residues, with a consequent drop to 1.8 of the typical ratio between C4 and C6 sulphate, that amounted to 8.1 in young HFs (Table 1A). Otherwise, the sulphation pattern of GlcN-GAGs in PGs extracted from the culture monolayers of young and senescent HFs did not significantly differ (Table 1B).

Resource column chromatography separated protein-free ^{3}H-labelled GAGs into three main fractions (Fig. 2, panels A). The percentage contribution of these fractions to total eluted radioactivity changed with cellular senescence: 18.4, 58.0 and 23.6 for a, b and c, respectively, in young HFs; 9.2, 69.5 and 21.3 for a, b and c, respectively, in senescent HFs. On the basis of their sensitivity to selective enzymatic digestions, fractions a and c contained GalN-GAGs and fraction b consisted only of GlcN-GAGs (Fig. 2, panels B and C). The relative proportion of fraction b increased with cellular senescence, well in agreement with the found higher resistance to Chase ABC and ACII digestion of total PGs extracted from late-passage HF cultures. The ratio of fraction c to fraction a increased in late-passage cultures, amounting to 1.2 and 2.3 in young and senescent HFs, respectively.

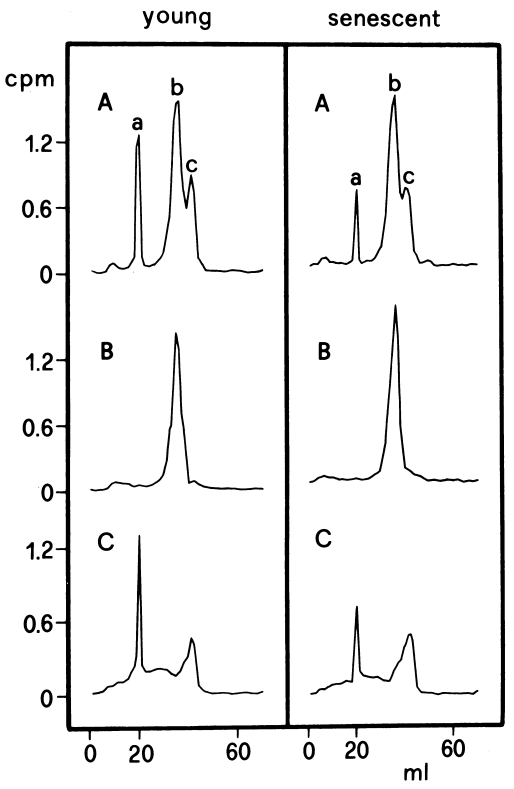

Fig. 2. Ion-exchange chromatography on Resource column of ^{3}H-labelled glycosaminoglycans of proteoglycans extracted from culture monolayers of young and senescent human skin fibroblasts. Determination of cpm is expressed in thousands. A: Samples without any treatment. B: Samples after chondroitinase ABC and ACII digestion. C: Samples after heparinase and heparitinase digestion. GAGs were freed of protein by papain digestion of ^{3}H-labelled PGs and subsequent removal of protease-resistant peptides by β-elimination (see Section 2).

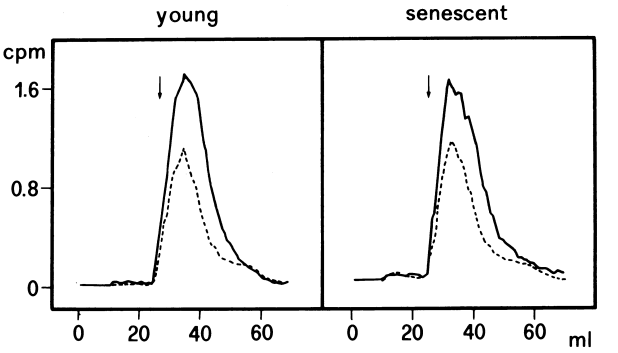

Fig. 3. Gel-filtration chromatography on Sephacryl S-300 column of ^{3}H-labelled glycosaminoglycans of proteoglycans extracted from culture monolayers of young and senescent human skin fibroblasts, before (solid line) and after (dotted line) chondroitinase ABC and ACII digestion. Determination of cpm is expressed in thousands. (↓) void volume. GAGs were freed of protein by papain digestion of ^{3}H-labelled PGs and subsequent removal of protease-resistant peptides by β-elimination (see Section 2).

HPLC analyses of the released unsaturated disaccharides revealed that fraction a contained unsulphated chains, whereas fraction c comprised sulphated GalN-GAGs, with a ratio C4- to C6-sulphation similar to that found for total PGs.

Gel-filtration chromatography profiles of protein-free GAGs from young and senescent HFs showed that their chain sizes were quite polydispersed and had similar distributions with about the same values of maximum frequency and of range (Fig. 3). This applied to both GalN-GAG and GlcN-GAG chains, as indicated by analyses run before and after Chase ABC and ACII digestion (Fig. 3).

4. Discussion

Some parameters of PGs isolated from HF monolayer cultures (the ability to assemble GAG chains, as assessed from [^{3}H]glucosamine incorporation, the total polyanionic charge and the size distribution of GAG chains) did not change with cellular senescence, in agreement with the findings reported for PGs secreted into the growth medium by the same cultures [10].

However, late-passage HFs synthesised a greater percentage of PGs containing GlcN-GAGs, which are known to be largely associated with the cell membrane as integral components [17]. This finding might well depend on the greater cell size of senescent HFs, which implies an increased development of outer cell membranes with a consequent higher production of their constituent macromolecules.

In late-passage cultures, the abilities of PGs to bind collagen type I and hyaluronic acid were significantly lowered. It should be recalled that GalN-GAG-containing PGs are mainly involved in these specific interactions [18,19]. Therefore, our finding might well be related to the structural modifications of GalN-GAG chains that were detected in senescent HFs and consisted in changes of their sulphation pattern. In particular, the molecules extracted from late-passage HF cultures displayed a much reduced ratio between C4- and C6-sulphated galactosamine residues. A marked decrease of this ratio was reported to occur also in the aging of human connective tissues, such as aorta [20], cartilage [21,22] and intervertebral disc [23]. Then, it is conceivable that cellular senescence and aging do affect the enzyme activities (sul-

photransferase and/or sulphatase) controlling both the presence and the position of sulphate groups on galactosamine residues of GAGs.

In contrast, the binding to laminin and fibronectin did not change in senescent HFs. These bindings concern mainly PGs with GlcN-GAGs [24,25], which are involved in a number of critical biological processes, such as cytokine action, cell adhesion and regulation of enzymic catalysis, all depending on protein-polysaccharide interactions mediated by specific saccharide sequence and positional sulphates [26]. Since gross structural modifications of GlcN-GAG chains from PGs were not observed in our HFs of late-passage cultures, it may be argued that the 'physiologic' process of cellular senescence could not tolerate relevant changes of GlcN-GAGs, greatly impairing individual cell viability.

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