Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1689 (2004) 152–161
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Matrix proteoglycans are markedly affected in advanced laryngeal squamous cell carcinoma

Spyros S. Skandalis[a], Achilleas D. Theocharis[a], Dimitrios A. Theocharis[b], Theodoros Papadas[c], Demitrios H. Vynios[a], Nickoletta Papageorgakopoulou[a,*]

[a] *Laboratory of Biochemistry, Section of Organic Chemistry, Biochemistry and Natural Products, Department of Chemistry, University of Patras, 26500 Patras, Greece*
[b] *Laboratory of Biological Chemistry, School of Medicine, University of Patras, Greece*
[c] *Department of Otolaryngology, University Hospital of Patras, Greece*

Received 14 November 2003; received in revised form 16 February 2004; accepted 12 March 2004
Available online 13 April 2004

Abstract

Proteoglycans (PGs) are implicated in the growth and progression of malignant tumors. In this study, we examined the concentration and localization of PGs in advanced (stage IV) laryngeal squamous cell carcinoma (LSCC) and compared with human normal larynx (HNL). LSCC and HNL sections were examined immunohistochemically with a panel of antibodies, and tissues extracts were analyzed by biochemical methods including immunoblotting and high performance liquid chromatography (HPLC). The results demonstrated significant destruction of cartilage in LSCC, which was followed by marked decrease of aggrecan and link protein. In contrast to the loss of aggrecan in LSCC, accumulation of versican and decorin was observed in the tumor-associated stroma. Biochemical analyses indicated that aggrecan, versican, decorin and biglycan comprise the vast majority of total PGs in both healthy and cancerous tissue. In LSCC the absolute amounts of KS/CS/DS-containing PGs were dramatically decreased about 18-fold in comparison to HNL. This decrease is due to the loss of aggrecan. Disaccharide analysis of CS/DSPGs from LSCC showed a significant reduction of 6-sulfated Δ-disaccharides (Δdi-6S) with a parallel increase of 4-sulfated Δ-disaccharides (Δdi-4S) as compared to HNL. The obtained data clearly demonstrate that tumor progression is closely related to specific alteration of matrix PGs in LSCC. The altered composition of PGs in cartilage, as well as in tumor-associated stroma, is crucial for the biological behaviour of cancer cells in the diseased tissue.

Keywords: Laryngeal cancer; Human; Proteoglycan; Sulfation pattern; Glycosaminoglycan

1. Introduction

Larynx is a uniquely complicated organ that is strategically located so that significant alteration of its anatomy can have a noticeable impact on vocal, respiratory, and swallowing physiology. The laryngeal cartilage, which is represented from four kinds of cartilage (cricoid, epiglottis, paired arytenoids, and shield-like thyroid cartilage), comprises the skeletal structure of the larynx [1]. The major extracellular matrix (ECM) components of cartilage are collagen type II and the large aggregating proteoglycan (PG), aggrecan. Aggrecan is consisted by an extended protein core onto which many chondroitin sulfate (CS) and keratan sulfate (KS) side chains are covalently bound. Cartilage contains also the two types of small size CS or/and dermatan sulfate (DS)-containing PGs, decorin and biglycan, which, in contrast to the large size aggrecan, carry only one or two side chains, respectively; the type of side chains varying with the tissue of origin. The small-sized PGs represent only 1–2% of the total mass of PGs in the cartilage [2–4].

Maintenance of the PG composition and content of cartilage is critical for the structural integrity and function of the tissue. One of the central pathophysiological features contributing to cartilage erosion during degenerative joint disease is the increased catabolism and loss of the large aggregating cartilage PG, aggrecan [5,6]. Degradation of cartilage involves proteolytic cleavage of both its major structural elements, namely aggrecan and type II collagen. Proteolysis and subsequent loss of the GAG rich

* Corresponding author. Tel.: +30-2610-997164; fax: +30-2610-997154.
E-mail address: papageorg.letta@chemistry.upatras.gr (N. Papageorgakopoulou).

0925-4439/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2004.03.006

region of aggrecan from cartilage is an early event in cartilage degeneration, while significant catabolism of the collagen fibrillar structure occurs later and may represent the point of irreversible cartilage damage [7].

Substantial experimental and clinical evidences suggest that the catabolism of ECM components is a prerequisite for invasive and metastatic behavior of solid tumors [8].

In a previous study [9], glycosaminoglycans (GAGs) from cancerous human laryngeal cartilage and normal adjacent tissue were isolated and characterized by means of enzyme susceptibility and high performance liquid chromatography (HPLC). It was shown that normal adjacent laryngeal tissue contains all known GAG species, the content and composition of which were altered in laryngeal carcinoma. It is known that in advanced LSCC (stages III and IV) many of these lesions are associated with cartilage destruction and the tumor invades through it and/or extents into soft tissues of the neck [1,10]. This issue is an important one because destruction of the PG-rich ECM of cartilage in laryngeal cancer is likely to be a prerequisite for invasive and metastatic behaviour.

There is a lack of information on the metabolism of PGs in human laryngeal carcinoma. The aim of this study was to evaluate the state of matrix PGs in cartilage as well as in the tumor stroma in advanced LSCC. The results of this study, which is the first detailed study on this field, indicated significant compositional and structural alterations in PG components examined. The significant findings of this study were the dramatic loss of aggregate components, i.e. aggrecan and link protein, the altered sulfation pattern of the galactosaminoglycan (CS/DS) chains and the increased levels of peritumoral versican and decorin in LSCC.

2. Materials and methods

2.1. Chemicals

Monoclonal antibodies 6B6 (against the protein core of decorin), 2B1 (against the protein core of versican) and 5D4 (against KS) were purchased from Seikagaku (Japan). Monoclonal antibodies 3B3 (against chondroitin/dermatan-6-sulfate stubs), 2B6 (against chondroitin/dermatan-4-sulfate stubs), 9/30/8A4 (against an epitope located at the C-terminal part of link protein), PR 8A4 (against biglycan) and 1C6 against the hyaluronan binding region of aggrecan were generous gifts of Prof. B. Caterson (University of Cardiff, UK). Polyclonal antibodies raised in rabbits against aggrecan and link protein were prepared in our laboratories. Goat anti-rabbit and anti-mouse IgG, both conjugated with peroxidase, guanidine HCl (GdnHCl) grade I, cesium chloride (CsCl), benzamidine–HCl, phenylmethylsulfonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), 6-aminohexanoic acid, cetylpyridinium chloride (CPC), were from Sigma Chemical Co. (St. Louis. MO, USA). Moreover, 2× crystallized papain (EC 3.4.22.2), chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4), chondroitinase AC II from *Arthrobacter aurescens* (EC 4.2.2.5), keratanase from *Pseudomonas* sp. (EC 3.2.1.103) and DEAE-Sephacel were from Sigma. All other chemicals used were of the best grade commercially available.

2.2. Analytical methods

The sulfated GAG (SGAG) content of isolated total PGs and purified galactosaminoglycan-containing PGs (GalAGPGs) was estimated by using the metachromatic dye 1,9-dimethyl methylene blue [11]. Total PGs and GalAGPGs content, expressed as CS equivalents per wet weight of tissue, were derived (without correction for KS or other components contributing to the change in absorbance) from a calibration curve created using whale CS as a standard. The amounts and types of the unsaturated disaccharides released by chondroitinase ABC or AC II were determined by HPLC [12].

2.3. Tissue source

Specimens of human normal larynx (HNL) ($n=4$, 25–55 years old) were obtained from autopsies and frozen at −20 °C. The cancerous specimens were obtained from the larynx after total laryngectomy for laryngeal carcinoma. The specimens obtained from each patient were transferred to the laboratory, separated from surrounding tissues and used in the subsequent experiments. Eight patients with advanced laryngeal squamous cell carcinoma (LSCC) were included in the study. The specimens, containing cartilage and soft tissue, were classified according to UICC classification system and were characterized as advanced LSCC, stage IV. The Ethical Committee of the University Hospital of Patras approved the study design and informed consent was obtained from all patients before entry into the study. For biochemical analyses of the cancerous and normal laryngeal tissues, we separated cartilaginous and non-cartilaginous parts of the samples and we obtained aliquots of the tissues containing almost equal percentages of cartilaginous and non-cartilaginous tissues.

2.4. Extraction of PGs

For PG analysis, tissue aliquots (200–600 mg of wet weight of tissue) were extracted by gentle shaking for 24 h at 4 °C with 10 vol. of 4 M guanidinium HCl (GdnHCl). The GdnHCl solutions were buffered with 0.05 M sodium acetate pH 5.8 [13]. The extracting solution contained 0.01 M disodium EDTA, 0.005 M benzamidine–HCl, 0.1 M 6-aminohexanoic acid and 0.004 M PMSF as protease inhibitors [14]. NEM (0.01 M) was also included to avoid disulfide exchange [15]. The extraction mixture was filtered and the filtrate was retained. The residues were re-extracted

for an additional 24 h period with 10 vol. of the same extracting solution. The two extracts were then pooled and aliquots from each combined extract were used for various chemical analyses. Known amounts of residual tissues after double extraction were washed with water and then digested with papain and analysed for their chemical composition. The extracts were collected and stored at −20 °C for further experiments and analyses.

2.5. Isolation and purification of PGs

In order to isolate total PGs from each specimen, equal parts from each specimen extract were subjected to precipitation with 5 vol. of ethanol. The isolated PGs were dissolved in 10 M formamide–0.05 M sodium acetate, pH 6.0, containing 0.2% (v/v) Triton X-100 and the proteinase inhibitors and were applied on to a DEAE-Sephacel column, which was equilibrated with the same buffer [16]. HA was eluted by washing of the column with 3 vol. of the same buffer containing 0.3 M NaCl. PGs were fractionated by elution of the column with 10 vol. of a linear gradient ranging from 0.3 M–0.85 M NaCl in formamide buffer [17]. Fractions obtained with 0.4–0.5 M NaCl and 0.55–0.7 M NaCl corresponded to heparan sulfate PGs (HSPGs) and GalAGPGs, respectively, confirmed by chemical and electrophoretic analyses as described before [17]. GalAGPG fractions were pooled and concentrated for further experiments. HSPG fractions were not further examined in this study.

2.6. Enzymatic degradations

Digestions with papain were performed at 60 °C for 18 h (0.2 mg of papain/g wet weight of tissue) in 0.1 M Tris–HCl, pH 7.2, containing 0.01 M disodium EDTA and 0.005 M cysteine–HCl. For chondroitinase ABC or chondroitinase AC II digestions, solutions of GAGs or PGs (1 mg of uronic acid/ml) in 0.1 M Tris–acetate, pH 7.3, were incubated at 37 °C for 4 h in the presence of 0.1 unit of chondroitinase ABC or AC II per milliliter. For keratanase digestion, the samples were incubated at 37 °C for 4 h in 0.1 M Tris–HCl, pH 7.4, in the presence of 0.1 unit of keratanase per micromole of galactose. Heating the solutions at 100 °C for 3 min terminated the digestions.

2.7. SDS/PAGE and western blotting

Polyacrylamide gel electrophoresis (PAGE) was performed on gels of linear gradient concentration of acrylamide (4–20%) according to the method of Laemmli [18]. Samples of isolated PGs, as well as tissue extracts derived from equal wet weight of tissues, were treated with chondroitinase ABC and/or keratanases and

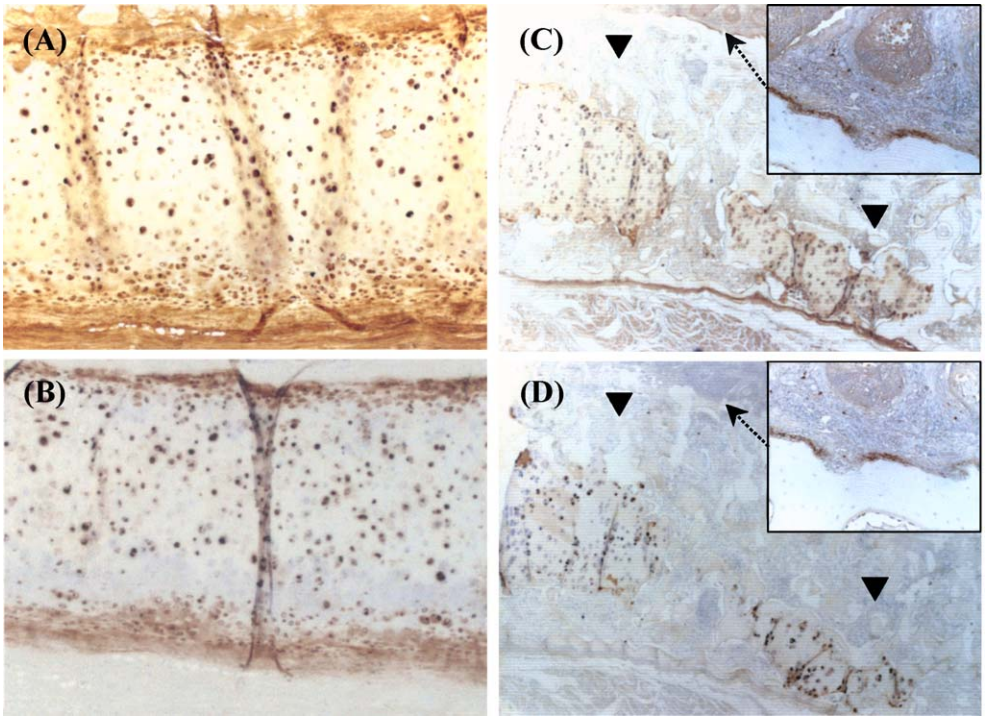

Fig. 1. Localization of aggrecan (A and C) and link protein (B and D) in HNL (A and B) and LSCC (C and D). Sections were stained using either a polyclonal antibody against aggrecan or 1C6 monoclonal antibody. Link protein was immunolocalized using 9/30/8A4 monoclonal antibody. Original magnification × 50 (A–D). The destruction and ossification of the cartilage is showed by the symbol ▼ in the corresponding specimens of LSCC. Insets indicate the mass of cancer cells in the vicinity of the ossified cartilage (original magnification × 250). (For color see online version).

were further electrophoresed and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P) at a constant current of 80 mA at 4 °C for 20 h in 0.05 M Tris–HCl, pH 8.3. The membranes were washed with 0.14 M NaCl in 0.01 M phosphate buffer, pH 7.2, containing 0.1% (v/v) Tween-20 (PBS-T) and blocked with 5% dry defatted milk (DDM) in PBS-T. They were then immersed in antibody solution against either versican (2B1), decorin (6B6), link protein (9/30/8A4), biglycan (PR 8A4), KS (5D4), chondroitin/dermatan-6-sulfate stubs (3B3), chondroitin/dermatan-4-sulfate stubs (2B6), diluted 1:2000 or aggrecan diluted 1:5000 in PBS-T–1% DDM and incubated for 1 h at room temperature. After repeated washings with PBS-T, the membranes were immersed in the appropriate second antibody diluted 1:5000 in PBS-T–1% DDM, incubated for 1 h at room temperature and washed exhaustively with PBS-T. The immunoreacting bands were visualized by using the ECL reagents (Amersham, UK) according to the manufacturer's instructions and by exposure to Agfa Curix X-ray film. Exposure varied from 1 to 30 min, depending on the experiment.

2.8. Immunohistochemistry

Specimens processed for light microscopy were fixed in 4% (v/v) buffered formaldehyde for 40 h at 4 °C, embedded in paraffin and sections of 5 μm were taken. Following dewaxing and rehydration, the sections were treated with 1 unit/ml chondroitinase ABC for 15 min at 37 °C to detach GAG chains from the protein core. Endogenous peroxidase activity was quenched with 3%

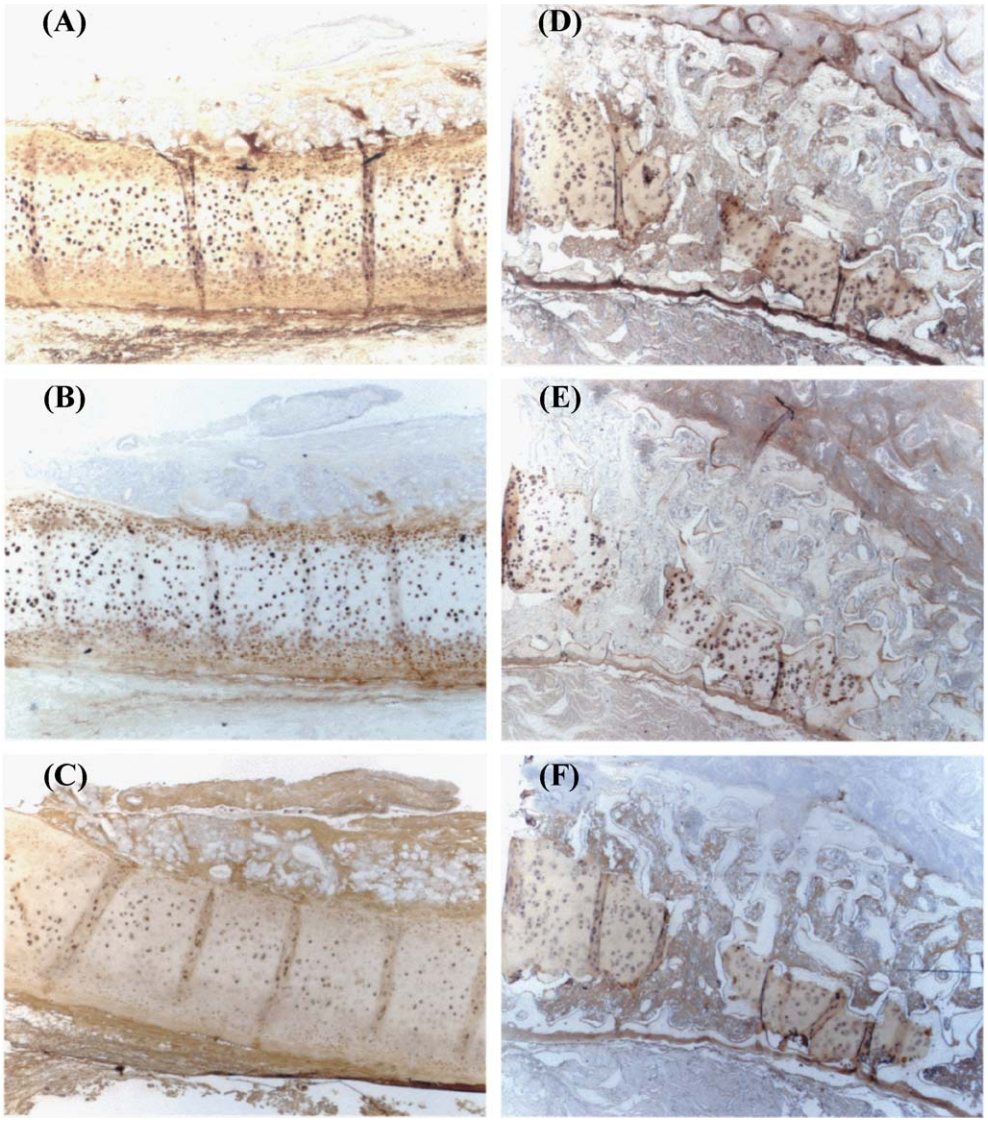

Fig. 2. Localization of 6-sulfated stubs (A and D), 4-sulfated stubs (B and E) and KS (C and F) in HNL (A, B and C) and LSCC (D, E and F). Sections were stained using 3B3, 2B6 and 5D4 monoclonal antibodies against 6-sulfated, 4-sulfated stubs and KS, respectively. Original magnification × 25 (A–C) and × 50 (D–F). (For color see online version).

(v/v) hydrogen peroxide for 5 min at room temperature. Nonspecific antibody binding was blocked by incubation with 3% (v/v) normal rabbit or swine serum in PBS for 20 min at room temperature. The slides were incubated with primary antibodies (polyclonal anti-aggrecan, 1:500; 1C6, 1:500; 9/30/8A4, 1:500; 5D4, 1:4000; 6B6, 1:1000; 2B1, 1:1000; 2B6, 1:2000; 3B3, 1:333) diluted in PBS containing 1% (v/v) normal rabbit or swine serum overnight at 4 °C. The slides stained with 1C6 and 9/30/8A4 were reduced and alkylated following incubation with 10 mM dithiothreitol in 50 mM Tris–HCl–200 mM NaCl, pH 7.4, for 2 h at 37 °C and 40 mM iodoacetamide in PBS for 1 h at 37 °C, respectively, before the treatment with chondroitinase ABC. The obtained antigen–antibody complexes were visualized by 30-min incubation at room temperature using biotinylated rabbit anti-mouse antibody or biotinylated goat anti-rabbit antibody diluted 1:200 and the avidin-biotin peroxidase technique (Dakopatts) according to manufacturer's instructions. The staining was developed with 3,3-diaminobenzidine (DAB)/hydrogen peroxide for 5 min at room temperature and slides were counterstained with hematoxylin.

2.9. Statistical analysis

Statistically significant differences were evaluated by *t*-test using the microcall origin software (version 3.2).

3. Results

3.1. Immunohistochemical studies

We examined the distribution of PGs, GAG chains, disaccharide stubs of GAG chains and link protein in HNL and LSCC. Both normal and neoplastic epithelia were negative for the presence of aggrecan and link protein (figure not shown) indicating that epithelial and fibroblast cells do not express aggrecan and link protein (aggregate components). In contrast, the loose connective tissue surrounding cartilage and cartilage of HNL and LSCC showed various degrees of immunoreactivity with all the antibodies tested. Strikingly, the immunostaining of the aggregate components in HNL (Fig. 1A and B, respectively) was different from that in LSCC (Fig. 1C and D, respectively), displaying significantly de-

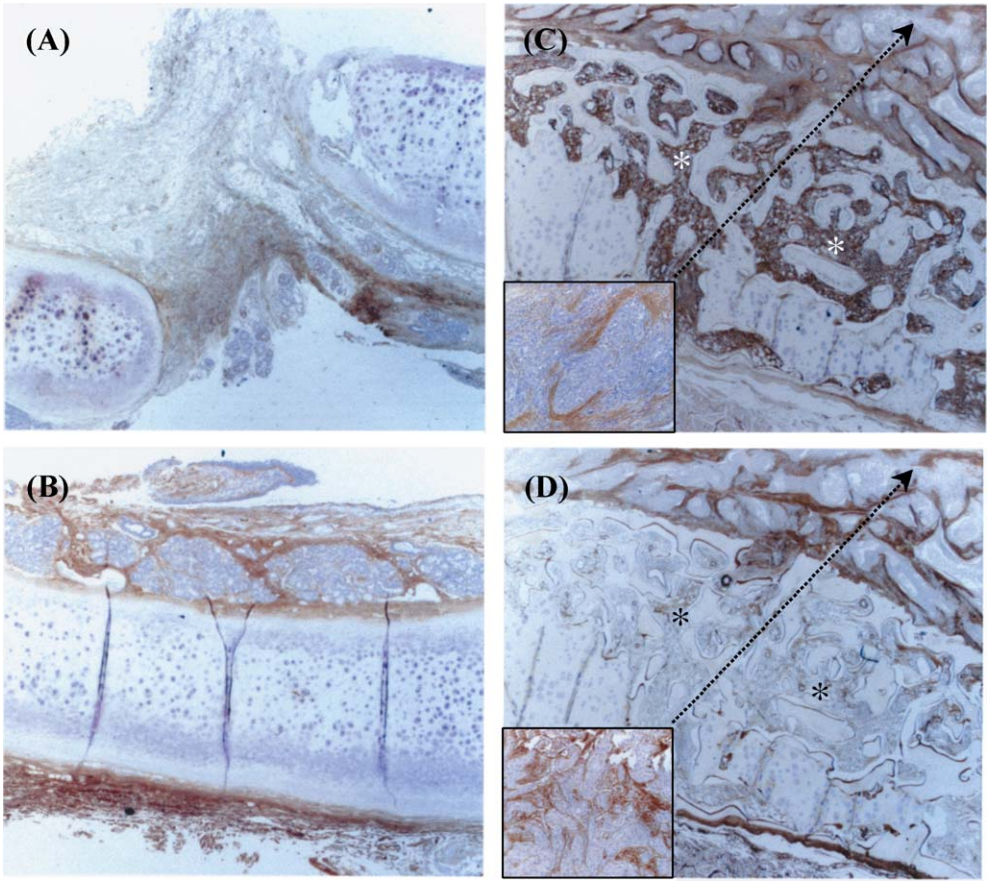

Fig. 3. Versican (A and C) and decorin (B and D) immunolocalization in HNL (A and B) and LSCC (C and D) was examined using 2B1 and 6B6 monoclonal antibodies, respectively. Original magnification ×25 (A and B) and ×50 (C and D). Asterisks indicate the staining of versican and decorin in the newly formed matrix in the destructed and ossified cartilage in the corresponding specimens. Insets show the intratumorous localization of versican and decorin in the cancerous tissue surrounding the destructed and ossified cartilage (original magnification ×250). (For color see online version).

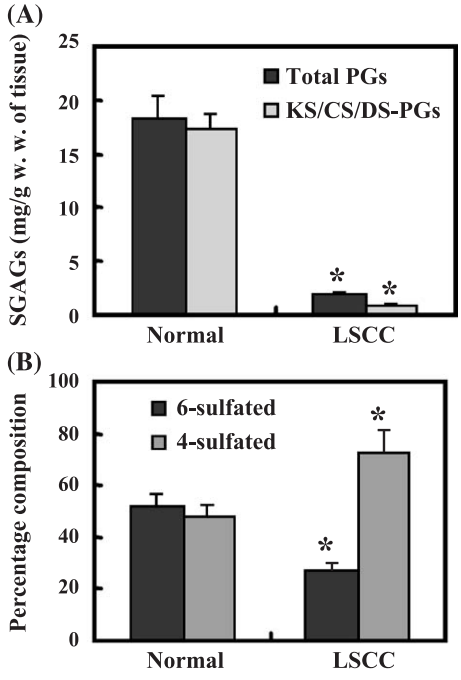

Fig. 4. (A) Quantification of total and KS/CS/DS-containing PGs in LSCC and HNL. The amounts of PGs are expressed as milligrams of sulfated GAGs per gram of wet weight of tissue. (B) Percentage composition of disaccharides present on CS/DS-PGs in LSCC and HNL. Values are the mean ± S.D. *$P \leq 0.05$.

creased levels of aggrecan and link protein in the peritumorous cartilage of LSCC when compared with those from normal cartilage in HNL. A similar distribution pattern with that of aggrecan in both HNL and LSCC was also observed for the chondroitin/dermatan 6-sulfate (Fig. 2A and D, respectively), chondroitin/dermatan 4-sulfate epitopes (Fig. 2B and E, respectively) and KS (Fig. 2C and F, respectively), since these epitopes and KS are intrinsic components of the aggrecan molecules.

These CS/DS stubs and KS showed a marked reduction in the peritumorous cartilage of LSCC in comparison to HNL following the decrease of aggrecan in the diseased tissue. As shown by the distribution of the major components of the cartilage, the advanced LSCC is associated to marked destruction of cartilage (Figs. 1 and 2). The latter is often accompanied by ossification and invasion by inflammatory and/or cancer cells (Figs. 1 and 2).

The use of 2B1 and 6B6 monoclonal antibodies, which recognize versican and decorin, respectively, showed a different distribution of these PGs between normal and neoplastic tissues. Decorin and versican are located in the loose connective tissue between epithelial cells and cartilage in HNL. Decorin exhibited a more intense reactivity in comparison to versican and was far more prominent in perichondrium (Fig. 3A and B). In LSCC, prominent immunostaining for versican and decorin was observed in stroma (Fig. 3C and D) associated with malignant areas of sectioned laryngeal tissue, whereas lower amounts of versican and decorin were identified in stroma surrounding nonmalignant tissues. Furthermore, a strong immunostaining for versican but not for decorin was found in the newly formed matrix of the remodeled- ossified cartilage in LSCC.

3.2. PG analysis

The concentration of total isolated and purified by anion-exchange chromatography PGs from each normal and cancerous specimen was estimated based on the content of SGAGs. The elution profiles from DEAE-Sephacel column

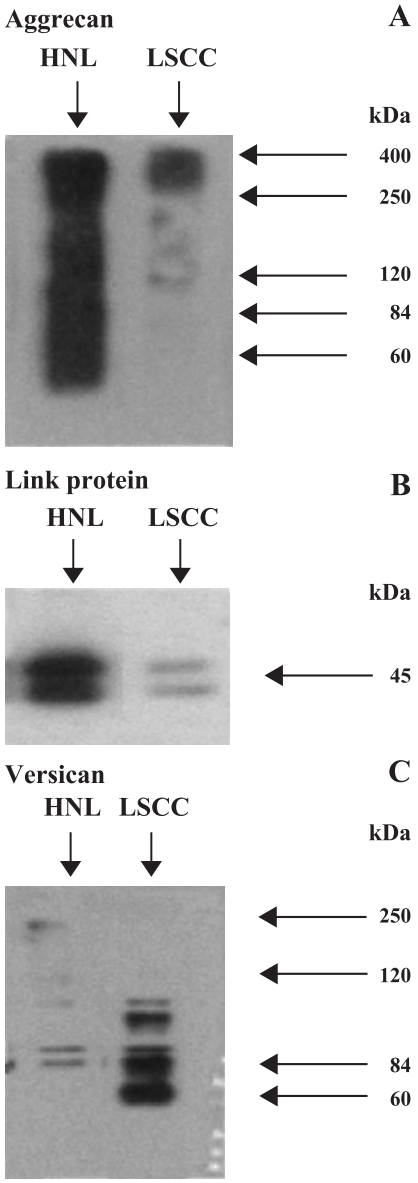

Fig. 5. Immunoblot analyses of HNL and LSCC for aggrecan (A), link protein (B) and versican (C). Isolated PGs were treated with chondroitinase ABC and keratanases, electrophoresed on 4–20% SDS-PAGE and blotted on membranes. The membranes were probed with polyclonal antibody against aggrecan and 2B1 monoclonal antibody against versican. Tissues extracts were also analysed for the presence of link protein using 9/30/8A4 monoclonal antibody. Figure indicates a representative case.

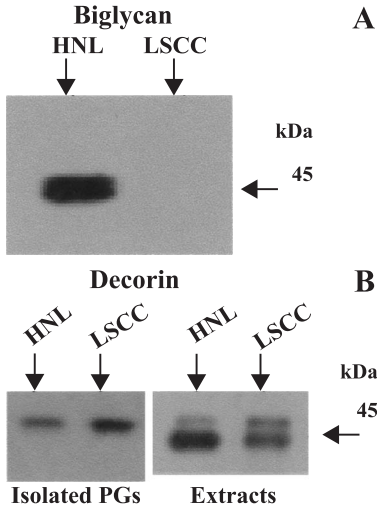

Fig. 6. Immunoblot analyses of HNL and LSCC for biglycan (A) and decorin (B). Isolated PGs were treated with chondroitinase ABC and keratanases, electrophoresed on 4–20% SDS-PAGE and blotted on membranes. The membranes were probed with PR 8A4 and 6B6 monoclonal antibodies against biglycan and decorin, respectively. Tissues extracts were also analysed for the presence of non-glycanated forms of decorin. Figure indicates a representative case.

(figures not shown) indicated that the vast majority of total PGs was KS/CS/DS-containing PGs and was eluted with 0.55–0.7 M NaCl. The quantitative data (Fig. 4A) showed a dramatic decrease of the absolute amounts of both total PGs and KS/CS/DS-PGs in LSCC, about 9-fold and 18-fold, respectively, in comparison to HNL.

Isolated PGs from each normal and cancerous specimen were exhaustively digested with chondroitinase ABC, which totally degrades the galactosaminoglycan (CS/DS) chains. HPLC analysis of the CS/DS-derived Δ-disaccharides indicated that Δdi-6S and Δdi-4S disaccharides represent 52% and 48%, respectively, of chondroitinase ABC derived disaccharides in samples from HNL (Fig. 4B). The proportion of Δdi-6S was significantly decreased from 52% to 27% in LSCC, indicating that Δdi-4S disaccharides predominated in LSCC. Traces of non-sulfated Δ-disaccharides were detected in the PG digests with chondroitinase ABC from both normal and cancerous specimens.

3.3. Western blot analyses

Aliquots of PG digests from each specimen, treated with chondroitinase ABC and/or keratanases, were examined for the presence of various types of PGs using a series of antibodies.

Intact or fragmented aggrecan in PG digests was investigated by using a polyclonal anti-aggrecan antibody. A typical result is presented in Fig. 5A. All PG digests showed a diversity of reactive material ranging from 60 to over 250 kDa, corresponding to almost free G1 and full-length core protein, respectively. As expected, significant decrease in reactivity against aggrecan was observed in LSCC in comparison to that of healthy. Exactly the same results were observed in the case of link protein, which was immunolocalised with 9/30/8A4 antibody (two major splice isoforms) (Fig. 5B).

The presence of intact or fragmented versican in the PG digests was investigated in a similar manner (Fig. 5C). It was found that versican was present in all PG digests. It is also noteworthy that LSCC contained relative higher amounts of versican with epitopes of lower molecular size than healthy tissue. In HNL, reactivity for versican was observed in two bands with Mr>250 kDa, but lower-

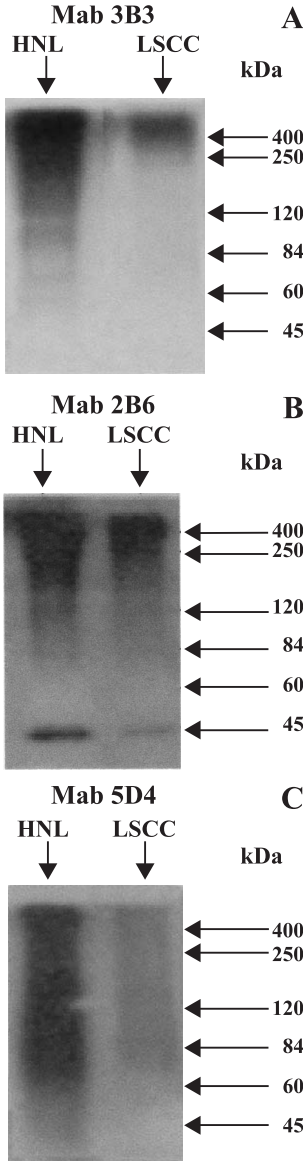

Fig. 7. Immunoblot analyses of HNL and LSCC for 6-sulfated stubs (A), 4-sulfated stubs (B) and KS (C). Isolated PGs were treated with chondroitinase ABC and keratanases (A and B), or only with chondroitinase ABC (C), electrophoresed on 4–20% SDS-PAGE and blotted on membranes. The membranes were probed with 3B3, 2B6 and 5D4 monoclonal antibodies against 6-sulfated, 4-sulfated stubs and KS, respectively. Figure indicates a representative case.

molecular size bands were also found. The above mentioned as well as other lower-molecular size bands predominate in LSCC suggested that they represent cleavage products of the intact versican core proteins.

Biglycan was identified in HNL but it was absent or traces were detected in LSCC (Fig. 6A). Decorin was also identified in varying degree of reactivity in both HNL and LSCC (Fig. 6B). Immunoblotting of extracts from cancerous and healthy tissues without purification of PGs showed that both tissues express two core proteins of decorin, the lower band corresponding to non-glycanated form of this PG.

PGs from normal and cancerous specimens were assessed for the presence of GAG stubs or KS by using monoclonal antibodies. Analyses with 3B3 monoclonal antibody are shown in Fig. 7A. Reactivity in molecules with a broad range of molecular size was observed in normal specimens. In contrast, in cancerous specimens significantly decreased levels of epitopes reactive with 3B3 antibody were observed, having high molecular size.

However, as was evident in Fig. 7B, positive immunostaining for lower-molecular size (less than 50 kDa) PG metabolites was observed in both normal and cancerous specimens using 2B6 antibody. In addition to aggrecan and versican metabolites, the protein band of ~45 kDa had electrophoretic mobility similar to that observed for decorin and biglycan core proteins (see Fig. 6A and B). These PGs seem to contain chondroitinase ABC-generated epitopes recognized by 2B6 antibody, indicating that their GAG chains are initiated with 4-sulfated disaccharide units.

The presence of KS on PG digests, which derived only following chondroitinase ABC, in both normal and cancerous specimens, was detected using 5D4 antibody (Fig. 7C). In normal and cancerous specimens, the KS-bearing PGs consisted a heterogeneous population, with profiles similar to those obtained for aggrecan (Fig. 5A). In LSCC, a significant decrease in KS content identical to that noticed for aggrecan was observed.

4. Discussion

Skeletal support of the larynx is provided by the hyoid bone and cartilages, which are bridged to each other by joints and membranes. The major cartilage of the larynx is the cricoid, thyroid, and the paired arytenoids cartilages. These major structural cartilages are all of hyaline type. A major component of the abundant ECM of hyaline cartilage is aggrecan. The epiglottis, in contrast, is composed of elastic cartilage containing numerous fenestrations.

Substantial experimental and clinical evidences suggest that the catabolism of ECM components is a prerequisite for invasive and metastatic behavior of solid tumors [8]. Histological studies have confirmed that the cartilages and membranes of the larynx provide natural barriers to the spread of endolaryngeal tumors [19]. Both the thyroid cartilage and its perichondrium appear to be effective barriers to the spread of cancer. The remodeling and destruction of ECM of laryngeal hyaline cartilages is a critical step to the spread and invasion of cancer. This concept is supported by the fact that tumor invades ossified portions of the laryngeal skeleton preferentially over cartilaginous portions. Two common sites of early ossification are the superior rim of the cricoid cartilage and the inferior rim of the thyroid cartilage. Consequently, cancer cells within the paraglottic space frequently invade these sites as it transgresses the cricothyroid membrane [19]. However, studies on the degeneration and destruction of the PG-rich ECM in LSCC have not yet been published.

In order to detect changes of ECM in LSCC in vivo, sections of HNL and LSCC were examined immunohistochemically for the presence of various types of PGs with a panel of monoclonal antibodies. As expected, strong immunoreactivity for aggrecan and link protein was observed in cartilage in sections of HNL. Significant reactivity was also observed for epitopes recognized by antibodies 3B3, 2B6 and 5D4, since 6-sulfated and 4-sulfated stubs of CS and KS comprise basic constituents of the aggrecan molecules. In comparison to HNL, sections of LSCC revealed an excessive reduction in reactivity for both aggregate components and aggrecan constituents of cartilage. In all cases of LSCC, a significant destruction of the cartilage was observed that was frequently accompanied by ossification. These histological features are compatible with the advanced stage of the lesion since they are critical for cancer spread as discussed above. The immunohistochemical findings were confirmed completely by Western blot analyses. The analyses with anti-aggrecan revealed that the range of size of the fragments or intact core proteins of aggrecan differed significantly between HNL and LSCC. In addition, analyses with antibodies 3B3, 2B6 and 5D4 showed that the distribution of the epitopes on core protein of aggrecan, which are reactive with these monoclonal antibodies, presented similar differences to those observed for aggrecan.

In contrast, versican and decorin, which consist a minor PG proportion in HNL, presented an increased reactivity in tumor-associated stroma in LSCC in comparison to HNL. The presence of versican and decorin in HNL was not surprising, since it has been reported that these PGs are present in the loose connective tissues of various organs [3,20]. In our study, the presence of non-glycanated form for decorin was detected in both HNL and LSCC. The presence of non-glycanated forms for decorin has also been observed in other tissues but their significance has not been documented yet [4]. Moreover, Western blot analysis for versican indicated that core proteins of versican are markedly fragmented in LSCC in comparison to HNL. Versican and decorin are abnormally expressed in a wide variety of malignant tumors.

Versican is a member of the hyalectan family and plays important roles in forming hyaluronan-rich matrix providing cells with an anti-adhesive environment [21–23] and

also supports cancer cell growth. Decorin is a powerful modulator of cell growth by affecting several key elements including matrix assembly, growth factor binding, and receptor tyrosine kinase activity [3,24–26]. Decorin levels are suppressed in most transformed cells, but markedly increased in the peritumorous stroma of several types of cancer. The latter may represent a natural biological response of the host cells to the invading neoplastic cells [3].

Biglycan was detected by Western blot analysis in HNL only. The negative immunoreactivity in LSCC does not signify the completely absence of biglycan in LSCC.

Quantification of the observed qualitative changes by detailed biochemical analyses indicated that the concentration of KS/CS/DS-PGs in LSCC decreased dramatically about 18-fold in comparison to HNL. Clearly, this decrease is due to aggrecan loss, since the other two PGs, versican and decorin, appear to increase. These dramatic changes imply that ECM of the laryngeal cartilages in LSCC undergoes significant degeneration, which is due to the decrease of aggrecan and link protein. The decrease of aggrecan in LSCC is thought to be mainly a consequence of increased degradation of this PG in the peritumorous cartilage, although the possibility for down-regulation of aggrecan biosynthesis in this tissue should not be excluded.

LSCC is characterized by altered sulfation pattern. An increase in the proportion of Δdi-4S in total extracted PGs was observed in LSCC in comparison to those from healthy samples. This finding is in accordance with other observations [9,27], and the results suggest tissue-specific biosynthetic responses in various types of cancers [28], since in rectum [29], colon [30], pancreatic [31] and gastric [17] adenocarcinomas, the proportion of Δdi-4S was found to be significantly decreased, as compared with that of the healthy tissue.

In conclusion, the increased catabolism of the cartilage PG aggrecan is a principal pathological process, which leads to the degeneration of laryngeal cartilage in LSCC. The consequent loss of CS and KS, which are intrinsic components of the aggrecan molecule, disturbs both the functional roles and structural integrity of the cartilage matrix. Over time, this process leads to irreversible cartilage erosion with the consequential result of the laryngeal cartilage losing its ability to behave as natural barrier to the spread and invasion of cancer. In situ degradation of aggrecan is a proteolytic process involving cleavage at specific peptide bonds located within the core protein. The mechanism of aggrecan degradation in LSCC comes as a provocative question that needs urgent answer.

Acknowledgements

We thank the Federation of European Biochemical Societies (FEBS) for supporting S. Skandalis with a short-term fellowship.

References

[1] V.T. De Vita, S. Helman, S.A. Rosenberg, Cancer Principles and Practice of Oncology, 6th edition, Lippincot Williams & Wilkins, Philadelphia, 2001, pp. 861–886.
[2] C.B. Knudson, W. Knudson, Cartilage proteoglycans, Semin. Cell Dev. Biol. 12 (2001) 69–78.
[3] R.V. Iozzo, Matrix proteoglycans. From molecular design to cellular function, Ann. Rev. Biochem. 67 (1998) 609–652.
[4] A.D. Theocharis, N.K. Karamanos, N. Papageorgakopoulou, C.P. Tsiganos, D.A. Theocharis, Isolation and characterization of matrix proteoglycans from human nasal cartilage. Compositional and structural comparison between normal and scoliotic tissues, Biochim. Biophys. Acta 1569 (2002) 117–126.
[5] T.E. Hardingham, A.J. Fosang, A.J. Dudhia, The structure, function and turnover of aggrecan, the large aggregating proteoglycan from cartilage, Eur. J. Clin. Chem. Clin. Biochem. 32 (1994) 249–257.
[6] B. Caterson, C.R. Flannery, C.E. Hughes, C.B. Little, Mechanisms involved in cartilage proteoglycan catabolism, Matrix Biol. 19 (2000) 333–344.
[7] T.E. Cawston, V.A. Curry, C.A. Summers, I.M. Clark, G.P. Riley, P.F. Life, J.R. Spaull, M.B. Goldring, P.J. Koshy, A.D. Rowan, W.D. Shingleton, The role of oncostatin M in animal and human connective tissue collagen turnover and its localization within the rheumatoid joint, Arthritis Rheum. 41 (1998) 1760–1771.
[8] M. Toriyama, A.E. Rosenberg, H.J. Mankin, T. Fondren, B.V. Treadwell, C.A. Towle, Matrix metalloproteinase digestion of aggrecan in human cartilage tumors, Eur. J. Cancer 34 (1998) 1969–1973.
[9] T.A. Papadas, M. Stylianou, N.S. Mastronikolis, N. Papageorgakopoulou, S. Skandalis, P. Goumas, D.A. Theocharis, D.H. Vynios, Alterations in the content and composition of glycosaminoglycans in human laryngeal carcinoma, Acta Oto-laryngol. 122 (2002) 330–337.
[10] T.E. Carey, J. Wennerberg, Cellular and molecular biology of the cancer cell, Head and Neck Pathology with Clinical Correlations, Churchill Livingstone, Philadelphia, 1998, pp. 3–15.
[11] R.W. Farndale, D.J. Buttle, A. Barrett, Improved quantitation and discrimination of sulfated glycosaminoglycans by use of dimethylmethylene blue, Biochim. Biophys. Acta 883 (1986) 173–177.
[12] N.K. Karamanos, A. Syrokou, P. Vanky, M. Nurminen, A. Hjerpe, Determination of 24 variously sulfated glycosaminoglycan and hyaluronan derived disaccharides by high-performance liquid chromatography, Anal. Biochem. 221 (1994) 189–199.
[13] S.W. Sajdera, V.C. Hascall, Protein polysaccharide complex from bovine nasal cartilage. A comparison of low and high shear extraction procedures, J. Biol. Chem. 244 (1969) 77–87.
[14] T.R. Oegema, V.C. Hascall, D.D. Dziewiatkowski, Isolation and characterization of proteoglycans from the Swarm rat chondrosarcoma, J. Biol. Chem. 250 (1975) 6151–6159.
[15] A.J. Aletras, C.P. Tsiganos, In situ interaction of cartilage proteoglycans with matrix proteins, Biochim. Biophys. Acta 840 (1985) 170–179.
[16] M. Yanagishita, V.C. Hascall, Characterization of low buoyant density dermatan sulfate proteoglycans synthesized by rat ovarian granulose cells in culture, J. Biol. Chem. 258 (1983) 12847–12856.
[17] A.D. Theocharis, D.H. Vynios, N. Papageorgakopoulou, S.S. Skandalis, D.A. Theocharis, Altered content composition and structure of glycosaminoglycans and proteoglycans in the gastric carcinoma, Int. J. Biochem. Cell Biol. 35 (2003) 376–390.
[18] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[19] G.J. Schwartz, B.L. Wenig, Clinical considerations for neoplasms of the larynx, Head and Neck Pathology with Clinical Correlations, Churchill Livingstone, Philadelphia, 1998, pp. 330–360.

[20] B. Bode-Lesniewska, M.T. Dours-Zimmermann, B.F. Odermatt, J. Briner, P.U. Heitz, D.R. Zimmermann, Distribution of the large proteoglycan versican in adult human tissues, J. Histochem. Cytochem. 44 (1996) 303–312.
[21] R.G. Le Barron, D.R. Zimmermann, E. Ruoslahti, Hyaluronate binding properties of versican, J. Biol. Chem. 267 (1992) 10003–10010.
[22] M. Yamagata, S. Suzuki, S.K. Akiyama, K.M. Yamada, K. Kimata, Regulation of cell–substrate adhesion by proteoglycans immobilized on extracellular substrates, J. Biol. Chem. 264 (1989) 8012–8018.
[23] M. Yamagata, K.M. Yamada, M. Yoneda, S. Suzuki, K. Kimata, Chondroitin sulfate proteoglycan (PG-M-like proteoglycan) is involved in the binding of hyaluronic acid to cellular fibronectin, J. Biol. Chem. 261 (1986) 13526–13535.
[24] E. Ruoslahti, Y. Yanagushi, Proteoglycans as modulators of growth factor activities, Cell 64 (1991) 867–869.
[25] R.V. Iozzo, The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins, J. Biol. Chem. 274 (1999) 18843–18846.
[26] M. Stander, U. Naumann, W. Wick, M. Weller, Transforming growth factor-beta and p-21: multiple molecular targets of decorin-mediated suppression of neoplastic growth, Cell Tissue Res. 296 (1999) 221–227.
[27] D.L. Uhlman, G.A. Niehans, Immunohistochemical study of chondroitin-6-sulphate and tenascin in the larynx: a loss of chondroitin-6-sulfate expression accompanies squamous cell carcinoma invasion, J. Pathol. 189 (1999) 470–474.
[28] A.D. Theocharis, D.A. Theocharis, High-performance capillary electrophoretic analysis of hyaluronan and galactosaminoglycan-disaccharides in gastrointenstinal carcinomas. Differential disaccharide composition as a possible tool-indicator for malignancies, Biomed. Chromatogr. 16 (2002) 157–161.
[29] M.E. Tsara, A.D. Theocharis, D.A. Theocharis, Compositional and structural alterations of proteoglycans in human rectum carcinoma with special reference to versican and decorin, Anticancer Res. 22 (2002) 2893–2898.
[30] A.D. Theocharis, Human colon adenocarcinoma is associated with specific post-translational modifications of versican and decorin, Biochim. Biophys. Acta 1588 (2002) 165–172.
[31] A.D. Theocharis, M.E. Tsara, N. Papageorgakopoulou, D.D. Karavias, D.A. Theocharis, Pancreatic carcinoma is characterized by elevated content of hyaluronan and chondroitin sulfate with altered disaccharide composition, Biochim. Biophys. Acta 1502 (2000) 201–206.