American Journal of Pathology, Vol. 138, No. 6, June 1991
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Alterations in Proteoglycan Synthesis Common to Healing Wounds and Tumors

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Wound healing and tumor stroma generation share several important properties, including hyperpermeable blood vessels, extravasation of fibrinogen, and extravascular clotting. In both, the deposits of fibrin gel serve initially as provisional stroma and later are replaced by granulation tissue. Proteoglycans (PG) are also important constituents of the extracellular matrix, but their composition and role in healing wounds and tumor stroma generation are poorly understood. The authors used immunohistochemical and biochemical methods to investigate the dermatan sulfate proteoglycan (DSPG) and chondroitin sulfate proteoglycan (CSPG) composition of healing skin wounds and solid tumors. By immunohistochemistry, the great majority of normal guinea pig and human dermis stained weakly for CSPG and strongly for decorin. In contrast, the granulation tissue of healing skin wounds and scars stained intensely for CSPG and weakly or not at all for decorin; however decorin staining was restored to normal intensity after digestion with chondroitin ABC lyase, suggesting that decorin antigenic sites had been masked by glycosaminoglycan (GAG) chains. Like wounds, the stroma of several carcinomas (line 1 guinea pig, human breast, colon, basal cell, and squamous) stained strongly for CSPG and weakly or not at all for decorin, but decorin staining developed after chondroitin ABC lyase digestion. Thus healing wounds and tumor stroma express a common pattern of altered PG staining, adding another to the properties these pathologic entities share. Proteoglycans extracted from healing wounds after* in situ *labelling with [^{35}S] Na sulfate contained more CSPG than normal dermis with significantly longer GAG chains. Granulation tissue also synthesized more DSPG than normal skin, with greater heterogeneity and longer GAG chains. These alterations in PG synthesis correlate with the cell proliferation, migration, and collagen synthesis that accompany wound healing and may provide clues to the mechanisms responsible for both wound healing and tumor stroma generation. (Am J Pathol 1991, 138:1437–1450)

Although initiated and propagated by different mechanisms, wound healing and tumor stroma generation share a number of important properties.[1] Both begin with spillage of plasma proteins, including fibrinogen and fibronectin. In both wounds and tumors, fibrinogen extravasates into the extravascular space, where it is rapidly clotted and cross-linked covalently to form a water-holding gel that also incorporates fibronectin. In both, the fibrin–fibronectin gel serves as a provisional matrix that provides a favorable substrate for the inward migration of macrophages, fibroblasts, and new capillaries. These last events are regulated at least in part by growth factors and perhaps other cytokines, which are released locally by platelets, inflammatory cells, and tumor cells.[2–4] With time, the fibrin–fibronectin gel is degraded and replaced, first by loose vascular connective tissue (granulation tissue) and later by dense, collagenous and relatively hypovascular and hypocellular connective tissue (designated scar tissue in wounds, desmoplasia in tumors).

In addition to fibrin, fibronectin, and collagen, proteoglycans (PG) represent another major structural component of connective tissues. Cultured fibroblasts synthesize several different PGs: versican, a large chondroitin sulfate proteoglycan (CSPG),[5] decorin, a small dermatan sulfate proteoglycan (DSPG)[6] as well as heparan sulfate proteoglycan (HSPG)[7] and perhaps other PG. Proteoglycans are also prominent components of basement membranes and commonly are associated with cell surfaces. Although regularly present in both healing wounds and tumors, the patterns of PG synthesis and functions have not been well defined in either process. In general, glycosaminoglycans (GAGs) are reported to be increased in tumors, and different tumors have been found to contain different levels of hyaluronic acid, HSPG, CSPG, and

Supported by NIH research grant CA 28471 and under terms of a contract from the National Foundation for Cancer Research.
Accepted for publication January 31, 1991.
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DSPG.[8] Moreover PG expression may be related to the malignant phenotype. Thus Chinese hamster ovary (CHO) mutants that synthesize ≤15% of the wild type HSPG are not tumorigenic.[9] In human breast tumors, DSPG content correlates well with the extent of tumor stroma fibrogenesis,[10] a finding consistent with the fact that the small DSPG, decorin, binds to collagen[11,12] and affects collagen fibrillogenesis and tensile strength.[13–15]

Proteoglycans also have been found to play important roles in several other cellular events that are central to both wound healing and tumor stroma generation, ie, cell adhesion, migration, and proliferation. Cell surface–associated PG act as receptors for other matrix molecules such as fibronectin.[16,17] Proteoglycans also may interact directly with the cellular cytoskeleton.[18] Cell-surface HSPG has been localized by immunocytochemistry to cell adhesion sites that coalign with microfilament bundles of the cytoskeleton.[19] Because of their highly charged nature it has been postulated that PG such as heparin and HSPG bind fibroblast growth factor and perhaps other growth factors, thereby protecting them from degradation and enhancing their activity.[20] In general, heparin and HSPG are thought to promote cell adhesion, whereas CSPG and DSPG decrease cellular adhesion to extracellular matrix components such as collagen and fibronectin.[2] Chondroitin sulfate proteoglycan constitutes a substantial fraction of the PG present in several types of tumors,[8] and their destabilizing effects on cell adhesion might be expected to facilitate tumor cell invasion and metastasis.

In view of the other known similarities between wound healing and tumor stroma generation, and because cell adhesion, migration, and proliferation are important events in both processes, we decided to determine whether any consistent patterns or alterations of PG synthesis or expression could be found in tumors and healing wounds compared with their corresponding normal tissues. Using an immunohistochemical approach, we demonstrated striking consistent changes in decorin and CSPG expression that are common to healing skin wounds and to the stroma of several types of guinea pig and human tumors. These immunohistochemical findings were confirmed and extended by isolation and preliminary characterization of the PG synthesized locally in healing guinea pig skin wounds.

Methods

Wounds and Tumors

Wounds were studied in female 250- to 300-g strain 2 guinea pigs. Skin wounds were bored with a 4-mm biopsy punch to the level of the panniculus carnosus on the shaved and depilated dorsal flanks of animals locally anesthetized with 0.2 ml 2% lidocaine (Invenex, Chagrin Falls, OH). Wounds were allowed to heal without covering.

To label newly synthesized PG with [^{35}S] sodium sulfate (Dupont, Wilmington, DE), 10 separate punch wounds were bored in the flanks of guinea pigs as above, except that animals were narcotized with Rompun (Xylazine, Haver-Lockhart Bayvet Division, Cutter Laboratories, Shawnee, KS) and ketamine hydrochloride (Parke-Davis, Morris Plains, NJ). Seven days later, when wound sites were filled with granulation tissue, 100 μl of 2.5 mCi/ml carrier free [^{35}S] sodium sulfate was injected directly into each wound site with a tuberculin syringe through a 30-gauge needle; 10 normal skin sites were similarly injected intradermally and marked with a Magic Marker for subsequent identification. A second set of [^{35}S] sodium sulfate injections was given 3 hours later and, after an additional 3 hours, guinea pigs were killed. The flank skin was removed as a single sheet, pinned out on dental wax, and [^{35}S] sodium sulfate–injected wound and control sites were removed individually with a 4-mm biopsy punch. Portions of some biopsies were fixed in paraformaldehyde-glutaraldehyde and processed for 1-μ epoxy resin (Epon) sections and autoradiography.[21] Insignificant amounts of radioactivity were detected in cetylpyridinium chloride (CPC) precipitates of the fixative solution. The majority of tissue was extracted for identification and characterization of PG (see below).

Line 1 bile duct carcinomas were passaged in ascites form in syngeneic strain 2 guinea pigs; solid tumors were generated by implanting 3×10^6 tumor cells subcutaneously in the dorsal flanks of other strain 2 animals.[22] At various intervals, tumors, wounds, and surrounding tissues were excised, fixed in 10% formalin, and embedded in paraffin for immunohistochemistry. Immunohistochemistry was also performed on unfixed, fresh-frozen tissues with similar results. Scar tissue isolated from five separate patients and tumors (at least five cases of each type) that had been removed at surgery for therapeutic purposes were obtained from the files of the Department of Pathology; all human tissues had been routinely fixed in formalin and embedded in paraffin.

Immunohistochemistry

A monoclonal antibody directed against chondroitin sulfate proteoglycan, MAb 938, was the gift of Dr. T. N. Wight; this antibody stains the CS chains of the large CSPG in several different tissues and species[23] (Yeo T-K, MacFarlane S, Wight TN, submitted for publication). A polyclonal rabbit antibody (anti-proteodermatan sulfate or PDS) was donated by Dr. H. Kresse; this antibody stains the core protein of a small DSPG[6,24]; this PG has

also been called PGII,[25] PG40,[26] and decorin[27] in a variety of human tissues. Although for the sake of convenience these antibodies are hereafter referred to as having reactivity against CSPG and decorin, their more restricted specificity, described above, must always be kept in mind.

Immunoperoxidase histochemistry was performed on 5-μ paraffin sections. For identification of CSPG, the primary monoclonal antibody was followed by either 1) a peroxidase-conjugated anti-mouse gamma G immunoglobulin (IgG; Biomeda, Corp., from Fischer Scientific), developing a red-colored precipitate with avidin-biotin complex (ABC; Biomeda, Corp.) or 2) a secondary biotinylated rabbit anti-mouse antibody (Vectastain kit, Vector Laboratories, Inc., Burlingame, CA), followed by incubation with avidin and then with biotinylated peroxidase and color development with diaminobenzidine (DAB, Polyscience, Inc., Warrington, PA), yielding a brown colored precipitate. Similar results were obtained with both methods. For identification of PDS, the primary rabbit anti-DSPG antibody was followed with a secondary swine anti-rabbit Gamma G immunoglobulin antibody (Dako Corp., Carpinteria, CA) and color was developed using the PAP method (Dako Corp.) with AEC or DAB. In some instances, tissue sections were digested for 20 minutes at 37°C with 0.5 U/ml of either chondroitin AC lyase or ABC lyase (Seikagaku Kogyo Co., Ltd., Tokyo, Japan) before immunohistochemical staining.

Isolation and Analysis of PG from [^{35}S] Na Sulfate-labeled Guinea Pig Skin and Wound Sites

Groups of 10 normal skin sites and 7-day healing skin wounds were pooled and minced in a tissue homogenizer in 8 ml of a buffer composed of 4 mol/l (molar) guanidine-hydrochloride, 50 mmol/l (millimolar) ethylenediaminetetra-acetic acid (EDTA), 50 mmol/l sodium acetate, 2% Triton X-100, 5 mmol/l benzamidine hydrochloride, 10 mmol/l *N*-ethylmaleimide, 1 mmol/l phenylmethylsulfonyl fluoride, and 100 mmol/l 6-aminohexanoic acid, pH 5.8. Minceates were then extracted for 24 hours in the same buffer at 4°C on a rotary shaker, pelleted by centrifugation at 9300*g* for 30 minutes at 4°C, and re-extracted in another 8 ml of the same buffer for an additional 24 hours at 4°C. First and second extracts were pooled and dialyzed against urea buffer (8 mol/l urea, 0.25 mol/l sodium chloride, 2 mmol/l EDTA, 0.3% Triton X-100, and 50 mmol/l TRIS, pH 7.5). Proteoglycans then were partially purified by chromatography on 3-ml Sephacel-diethylaminoethylcellulose columns, washing with urea buffer and then eluting with urea buffer–3 mol/l NaCl.

Incorporation of [^{35}S] sodium sulfate into PG was assayed by precipitation with CPC.[28] Briefly, 75-μl aliquots of [^{35}S]-labeled extract were spotted in duplicate on Whatmann 3-mm filter paper. Dried filter strips were washed five times for 1 hour each with 2 liters of 1% CPC in 0.05 mol/l sodium chloride to remove unincorporated label. Filter paper strips were dried again and retained radioactivity was determined by liquid scintillation counting.

The relative hydrodynamic sizes of the newly synthesized [^{35}S]-labeled proteoglycans were determined by Sepharose CL-4B gel chromatography in 4 mol/l guanidine-hydrochloride buffer with 0.5% Triton X-100 in 0.1 mol/l TRIS hydrochlroide, pH 7.0, followed by dialysis against TRIS buffer (20 mmol/l TRIS-hydrochloride, 0.2 mol/l sodium chloride, 1 mmol/l azide, pH 7.5) and precipitated with four volumes of 1.3% potassium acetate in 95% ethanol. The precipitates were reconstituted in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) buffer and analyzed on 7.5% SDS-PAGE.[29] The molecular weights of sodium borohydride–released GAG chains were estimated by chromatography on Sepharose CL-6B columns in TRIS buffer and compared with the data of Wasteson.[30] Ethanol-precipitated samples were dried, reconstituted in 1 mol/l sodium borohydride—0.05 mol/l sodium hydroxide and incubated for 20 hours at 45°C. Samples then were neutralized with glacial acetic acid, adding water to dissolve any precipitate, and dialyzed against water.

To identify the types of GAG chains present, portions of the dialyzed samples were digested with chondroitin ABC lyase (0.05 U/ml in 50 mmol/l TRIS buffer, pH 8.0, 35 mmol/l sodium acetate, 50 mmol/l sodium chloride, and 0.01% bovine serum albumin[31]), or chondroitin AC lyase (0.05 U/ml in 50 mmol/l TRIS buffer, pH 7.0[31]) for 4 hours at 37°C. Chain size was determined by Sepharose CL-6B gel chromatography.

Results

Distribution of CSPG (Large CS Chains) and Decorin (Core Protein) in Normal Skin and in Healing Skin Wounds

The skin punch biopsy model of wound healing in guinea pigs has been described previously.[22] Briefly, the wound defects quickly filled with clotted blood and plasma exudate, which became organized over a period of days by the ingrowth of fibroblasts, new blood vessels, and small numbers of mononuclear cells from the wound base and edges. In parallel, epidermis grew centripetally from the wound periphery over the developing granulation tissue, bridging the defect by 7 to 9 days. Thereafter the granulation tissue matured over a period of weeks to months

as fibroblasts and blood vessels decreased in number and collagen matrix increased.

The PG composition of healing skin was studied in duplicate samples taken from each of three guinea pigs at intervals of 2, 7, 14, 30, and 49 days after wounding and compared with that of normal skin. The great majority of the papillary and reticular dermis of normal guinea pig flank skin stained lightly or not at all with monoclonal antibody (MAb) 938 against CSPG (Figure 1A, B). Strong staining was observed in the papillae and connective tissue sheaths about hair follicles, however, in thin rims of connective tissue enveloping large and small blood vessels, and irregularly at the interface of the papillary dermis with the epidermis. In contrast, the antibody to decorin stained the great majority of the papillary and reticular dermis strongly and diffusely. The connective tissue of the papillae and sheaths around hair follicles, however, stained weakly or not at all for DSPG (Figure 1C).

The staining pattern of healing wounds differed strikingly from that of normal skin. At 7 or 14 days after wounding, the wound connective tissue stained intensely for CSPG and weakly or not at all for decorin (Figure 1D, E). At 4 weeks, decorin staining of wounds had increased slightly but remained reduced in comparison with normal

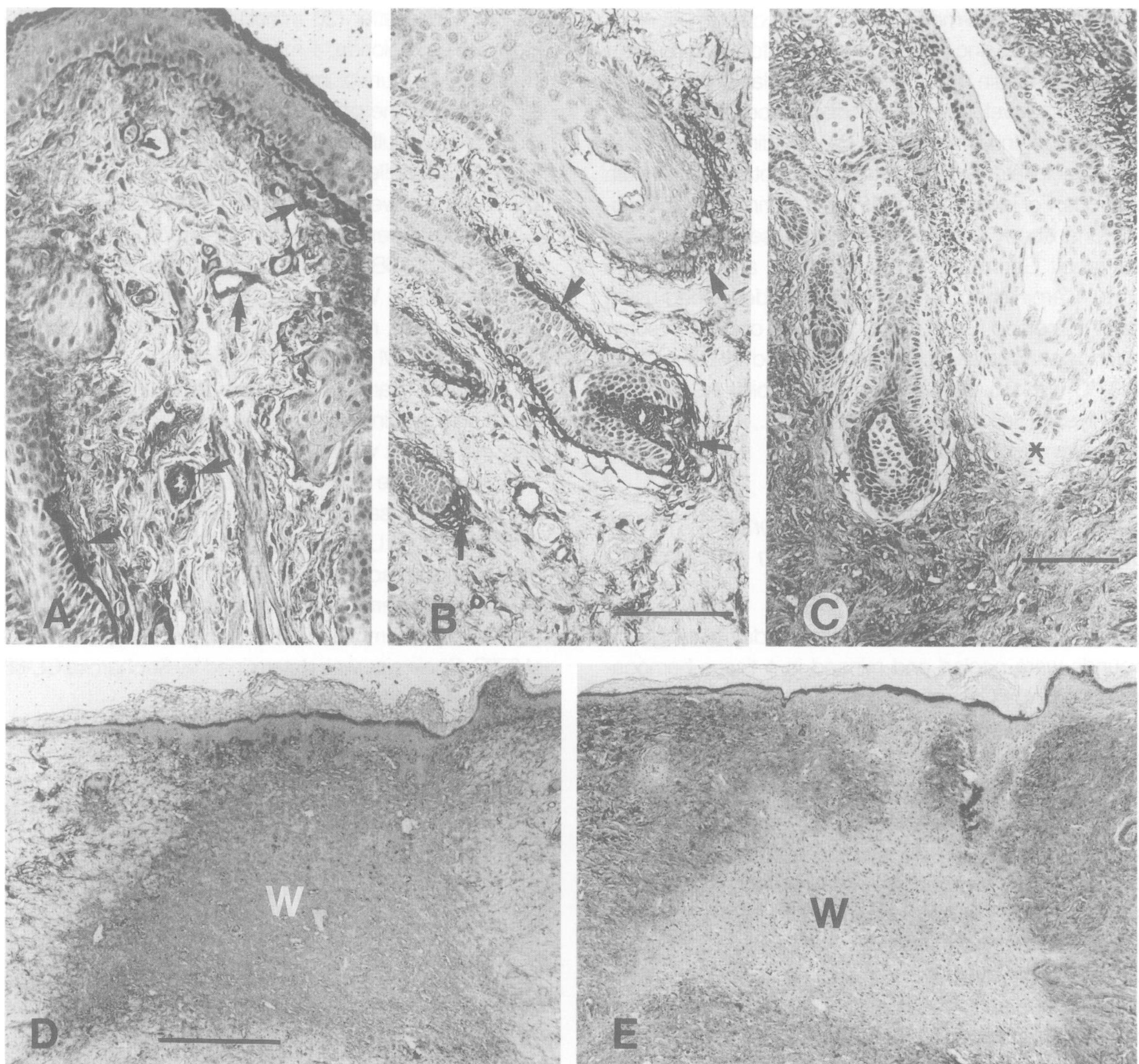


Figure 1. *Immunoperoxidase staining of normal guinea pig skin and of healing guinea pig punch biopsy wounds. Sections in this and in Figures 2 to 6 were counterstained with hematoxylin, which stains nuclei and epidermal surface keratin; all other black staining in black and white photomicrographs of histochemical sections represents antibody-specific immunohistochemical product. Normal papillary and reticular dermis stained weakly or not at all with MAb 938 to CS chains of CSPG, except for focal intense staining at the epidermal–dermal interface, in the dermal papillae and connective tissue sheaths associated with hair follicles and blood vessels (arrows,* **A** *and* **B***). In contrast, antibody to decorin core protein stained both the papillary and reticular dermis strongly but the dermal papilla and connective tissue sheaths of the hair follicles stained weakly or not at all (designated by *,* **C***). Healing 15-day guinea pig skin wounds exhibited a different pattern with strong staining of wound (W) for CSPG* **(D)** *but not for decorin* **(E)**. *The surrounding normal dermis stained with the reverse pattern. Color development with DAB. Bars:* **A–C**, *100 μ; D and E, 500 μ.*

flank skin; by contrast, CSPG staining of healing wounds remained significantly more intense than that of normal skin (not illustrated).

The patterns of antibody reactivity to CSPG and decorin in normal human skin were similar to those observed in the guinea pig. As in guinea pigs, healed human skin wounds (surgical scars of varying ages that had been resected from the trunk, extremities, scalp, and abdomen) all stained strongly for CSPG and weakly or not at all for decorin (Table 1).

Distribution of CSPG (Large CS Chains) and Decorin in Tumors

Line 1 bile duct carcinomas transplanted to the dermis and subcutaneous space of syngeneic stain 2 guinea pigs grow to form solid tumors containing abundant fibrous stroma.[22,32] In contrast to the surrounding normal dermis and subcutaneous tissue, line 1 tumor stroma stained much more intensely for CSPG and much less intensely or not at all for decorin (Table 1, Figure 2A, B).

Chondroitin sulfate proteoglycan and decorin staining was also studied in a number of different types of human tumors and the corresponding normal tissues from which each arose. In basal cell (Figure 3A, B) and squamous cell (not illustrated) carcinomas of the skin, tumor stroma stained intensely for CSPG and weakly or not at all for decorin, thus resembling the staining pattern of healing skin wounds.

Several distinct types of connective tissue are recognized in normal human breast, and these differed with respect to their staining pattern with the anti-CSPG and anti-decorin antibodies (Figure 4A, B, Table 1). The densely collagenous interlobular and interlobar connective tissues stained strongly for decorin and weakly for CSPG. In contrast, the looser intralobular connective tissue, most proximate to breast epithelium, gave the opposite pattern, staining intensely for CSPG and weakly or not at all for decorin. The stroma of all five invasive ductal breast carcinomas studied was strongly CSPG positive (Figure 4E, Table 1), except for occasional unstained collagen bundles, which permeated the stroma and which may have represented normal interlobular connective tissue that the tumor had invaded. Decorin staining of breast carcinoma stroma was uniformly weak or negative (Figure 4F).

The lamina propria of the normal large intestine (Figure 4C, D, Table 1) stained lightly for CSPG and very weakly or not at all for decorin. In all five carcinomas of the large bowel we studied, tumor stroma stained intensely for CSPG and very weakly or not at all for decorin (Figure 4G, H, Table 1).

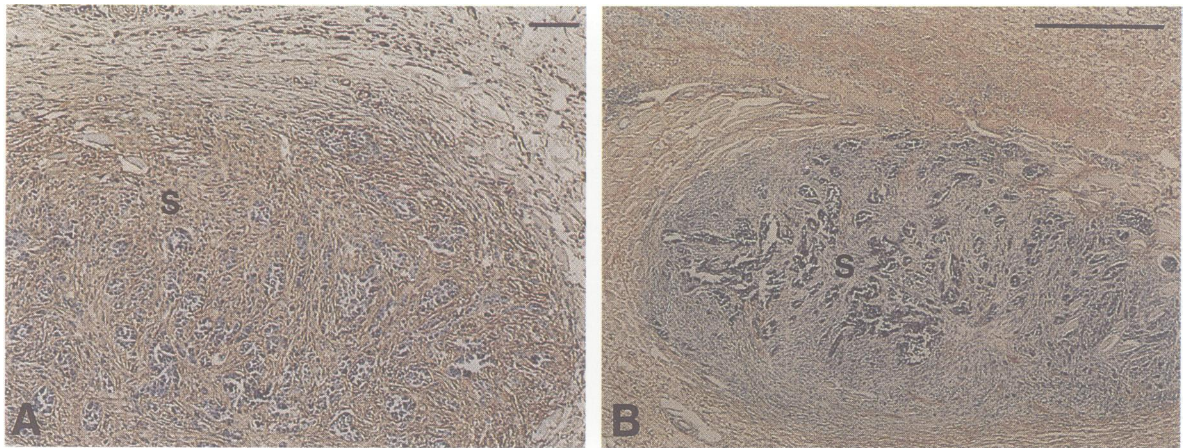
Effect of Chondroitin ABC Lyase Digestion on Decorin Staining

The immunohistochemical studies described above indicated that healing skin wounds, scars, and the stroma of a variety of tumors stained weakly or not at all with the antibody to decorin (Table 1). This lack of staining could reflect absence of the antigenic moiety, or, alternatively, masking of the epitope as by altered or increased numbers of GAG chains. To distinguish between these possibilities, tissue sections were incubated with chondroitinase ABC to digest dermatan and chondroitin GAG chains before immunohistochemical staining. After such digestion, wounds and tumor stroma stained strongly for decorin (Figure 5) and with approximately the same intensity as normal surrounding dermis. Similar predigestion with chondroitin AC II lyase, which digests chon-

Table 1. *Immunohistochemical Staining Patterns of Normal Connective Tissues, Wounds, and Tumor Stroma with Antibodies to CSPG and Decorin**

Tissue	Staining	
	CSPG	Decorin
Normal guinea pig or human skin		
Majority of papillary and reticular dermis	0–±	3+–4+
Discrete portions of the dermis (zones around hair follicles, other adnexae)	3+–4+	0–±
Granulation tissue and scars of healing guinea pig or human skin wounds	3+–4+	0–±†
Stroma of autochtonous or transplanted carcinomas growing in skin (guinea pig line 1, human basal cell or squamous cell)	3+–4+	0–±†
Connective tissue of normal human breast		
Intralobular	3+–4+	0–±‡
Interlobar and interlobular	±	3+–4+‡
Stroma of human breast carcinoma	3+–4+	0–±†
Lamina propria of normal human colon	1+–2+	0–±‡
Stroma of human colonic carcinoma	3+–4+	0–±‡

* Staining is designated 3+–4+ (strongly positive), 1+–2+ (positive), ± (weakly positive), or 0 (negative).
† Becomes strongly positive for decorin if immunohistochemical staining is preceded by digestion with chondroitin ABC lyase.
‡ Not tested for decorin staining after chondroitin ABC lyase digestion.



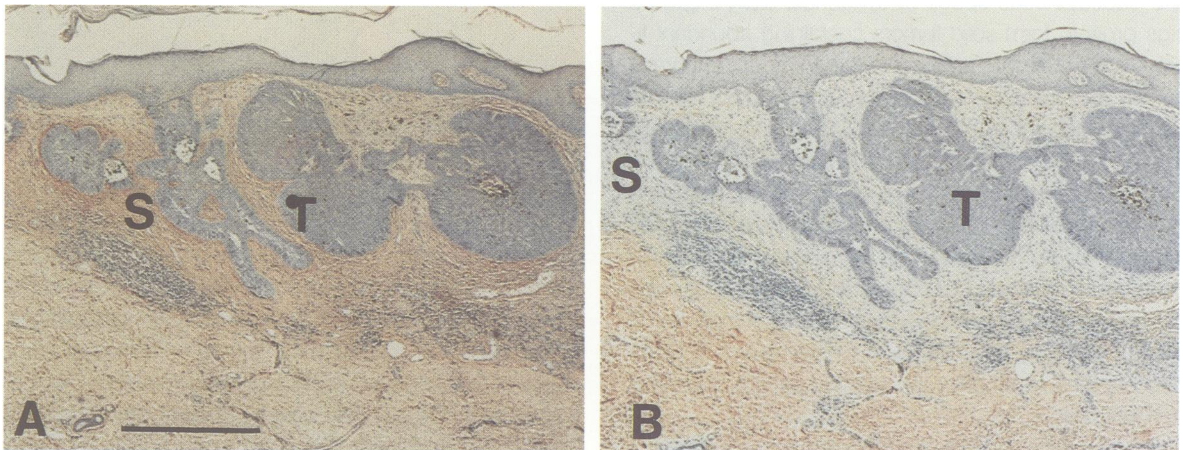


Figure 2. *Immunoperoxidase staining of line 1 bile duct carcinomas 6 days after transplant into the subcutaneous space of syngeneic strain 2 guinea pigs using DAB as substrate for color development (brown positive stain). Tumor stroma (S) stains strongly with MAb 938 (anti-CSPG)* (**A**) *but not with the antibody to decorin* (**B**). *In contrast, the surrounding normal connective tissues give the reverse staining pattern. Bars:* **A**, *100 μ;* **B**, *500 μ.*

Figure 3. *Immunoperoxidase staining of basal cell carcinoma (T) arising in human skin with MAb 938 to CSPG* (**A**) *and antibody to DSPG* (**B**). *The staining of the sections in this figure as well as in Figure 4 used AEC to develop the reddish positive color. Tumor stroma (S) stains intensely for CSPG and lightly or not at all for DSPG. The subjacent normal reticular dermis gives the reverse staining pattern, except for thin rims around blood vessels that are CSPG positive/decorin negative. Bars: 500 μ.*

droitin but not dermatan sulfate chains, also enhanced staining with anti-decorin antibodies, but to a lesser extent (not illustrated). These findings suggest that decorin is in fact present in healing wounds and in tumor stroma in substantial amounts, but in a form not recognized by our antibody without prior digestion of GAG chains.

Biochemical Characterization of PG Extracted from Normal Dermis and from Healing Wounds

The GAG of 7-day healing guinea pig punch biopsy wounds and normal flank skin were labeled by local intrawound or intradermal injections of [^{35}S] Na sulfate. Injected sites then were harvested and processed either for histology and autoradiography or extracted for biochemical characterization. Autoradiography of 1-μ Epon-embedded sections indicated that substantially more [^{35}S] Na sulfate was incorporated in the granulation tissue of healing wounds than in the immediately adjacent normal dermis (Figure 6). This impression was confirmed by quantitative analysis of [^{35}S] Na sulfate incorporation in healing wounds and in normal skin; CPC precipitates of wound extracts yielded nearly twice as much incorporated radioactivity per milligram wet weight as normal skin (13.4×10^6 dpm *versus* 7.2×10^6 dpm).

Labeled PG synthesized by healing wounds and normal dermis were partially purified by chromatography on Sepharose CL-4B columns. Extracts of normal skin yielded two major fractions that were pooled for studies, designated fraction II and fraction III (Figure 7). Extracts of 7-day wound sites differed from those of normal skin in several respects: 1) a new peak, fraction I, eluted with V_o;

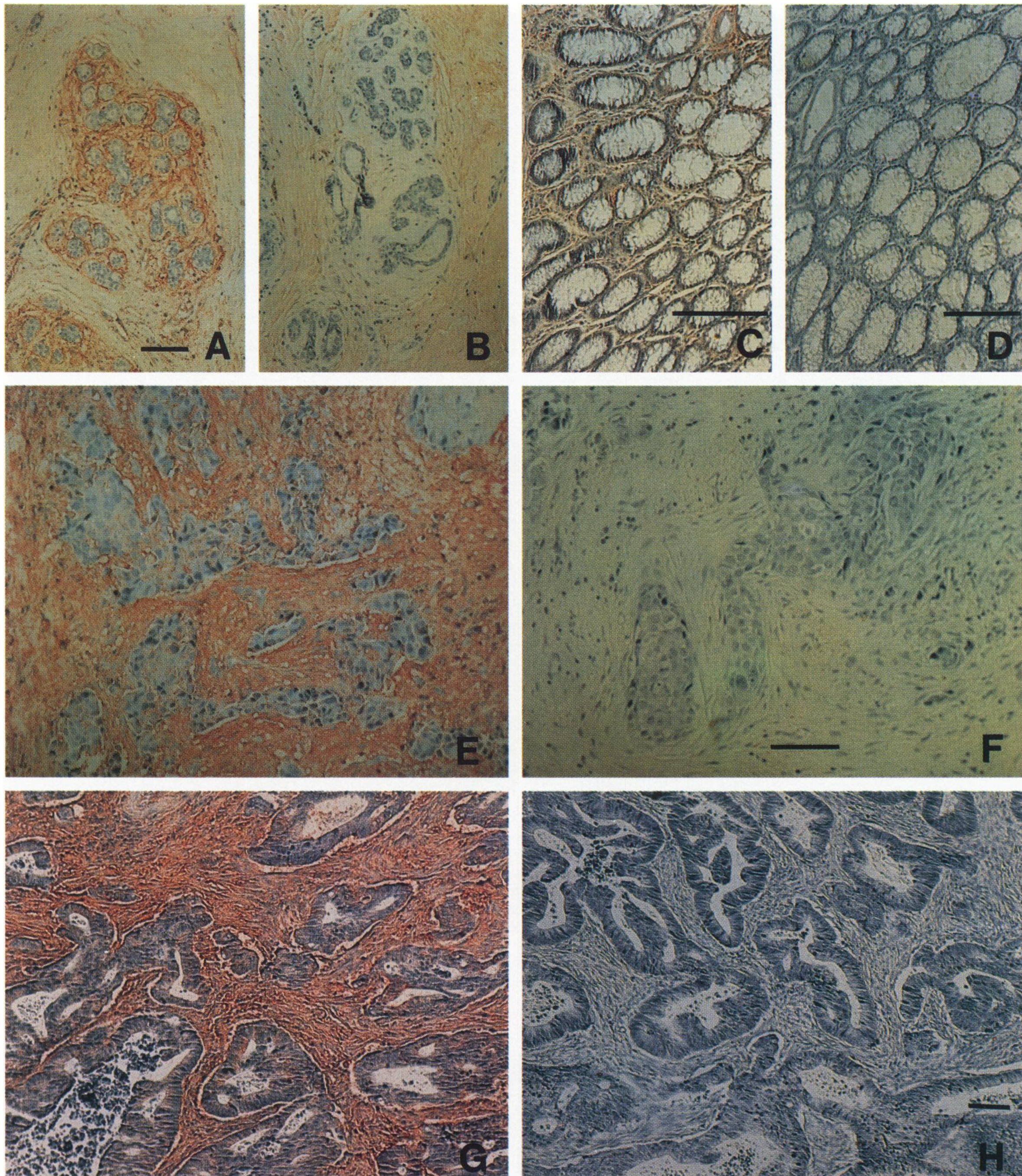


Figure 4. *Immunoperoxidase staining of normal human breast and colon and of carcinomas of human breast and colon.* **A, B:** *Intralobular connective tissue of normal breast stains strongly with MAb 938 to CSPG, whereas interlobular connective tissue does not* **(A)**. *The opposite pattern is observed with antibodies to decorin* **(B)**. **C, D:** *Lamina propria of normal colonic mucosa stains lightly with antibodies to CSPG* **(C)** *and not at all with antibodies to decorin* **(D)**. **E, F:** *Stroma of invasive ductal breast carcinoma stains intensely with MAb 938 to CSPG* **(E)** *but not with antibodies to decorin* **(F)**. **G, H:** *Stroma of human colon carcinoma stains intensely for CSPG* **(G)** *and not at all with antibodies to decorin* **(H)**. *Color development with AEC. Bars:* **A,B,E,F,G,H**, *all 100 μ;* **C** *and* **D**, *500 μ.*

2) fraction II was increased in absolute and relative amount; 3) the elution profile of fraction III shifted to the left from Kav = 0.54 (normal skin) to Kav = 0.49 (wound), indicating an increase in molecular size; and 4) fraction III peak of healing wounds was broader than that of control dermis, indicating greater size heterogeneity.

The larger size and greater heterogeneity of wound-derived fraction III were further substantiated by analysis on SDS-PAGE (Figure 8). The major band isolated from normal skin (Figure 8, lane 2), had an M_r of ~ 90,000 to 56,000 daltons and probably represents decorin based on its relative molecular weight[6,33] and because abun-

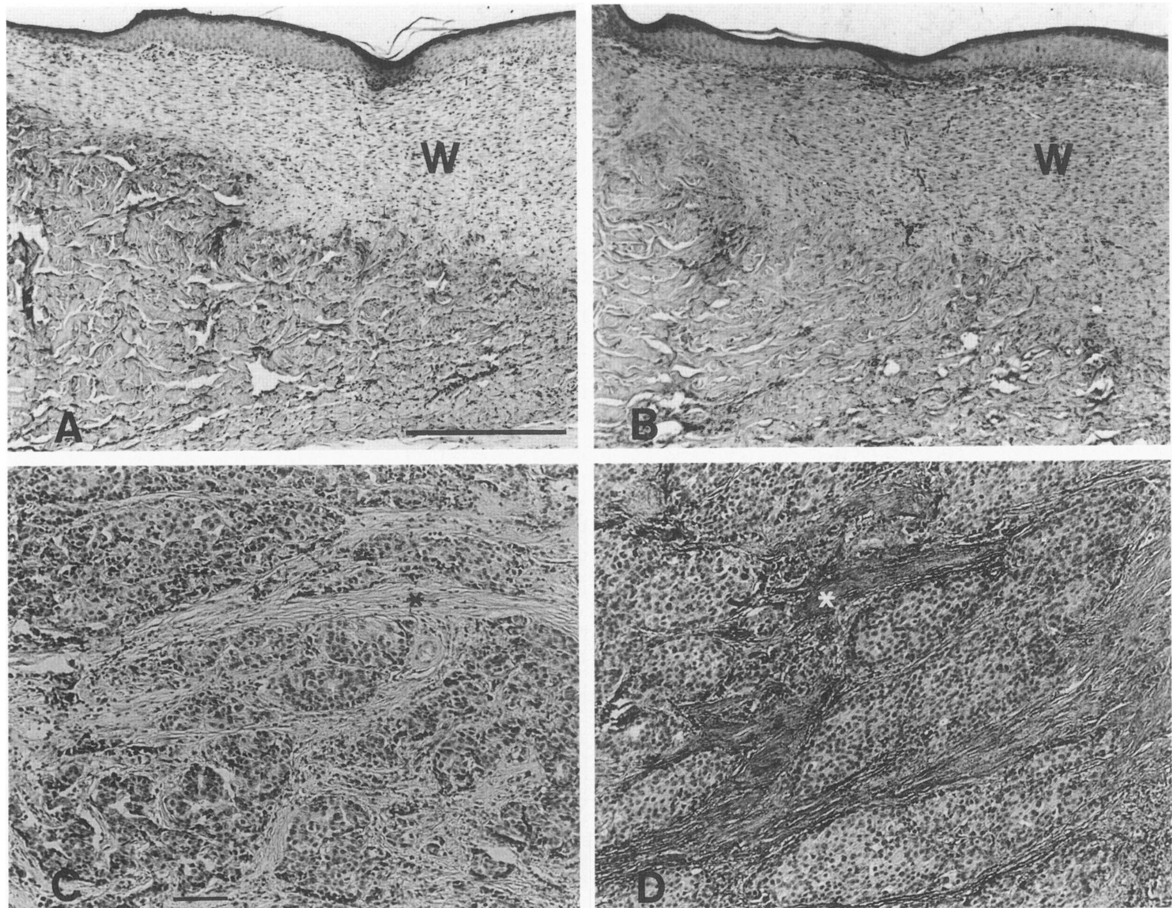


Figure 5. *Effect of chondroitin ABC lyase digestion on immunoperoxidase staining for decorin. Five-micrometer sections of formalin-fixed, paraffin-embedded guinea pig skin wound* (**A, B**) *and human breast carcinoma* (**C, D**) *were incubated with* (**B, D**) *or without* (**A, C**) *0.5 U/ml chondroitin ABC lyase for 20 minutes at 37°C before staining with anti-PDS (decorin) antibody. In* **A**, *wound stroma (W) of 15-day guinea pig wound exhibits little or no staining for decorin as compared with underlying normal reticular dermis. After digestion with chondroitin ABC lyase, the wound stroma and underlying dermis stain with approximately equivalent intensity* (**B**). *In* **C**, *stroma of human breast carcinoma (*) exhibits little or no staining with antibodies to decorin but, after digestion with chondroitin ABC lyase, the tumor stroma stains intensely with this antibody* (**D**). *Color development with DAB. Bars:* **A** *and* **B**, *500 μ;* **C** *and* **D**, *100 μ.*

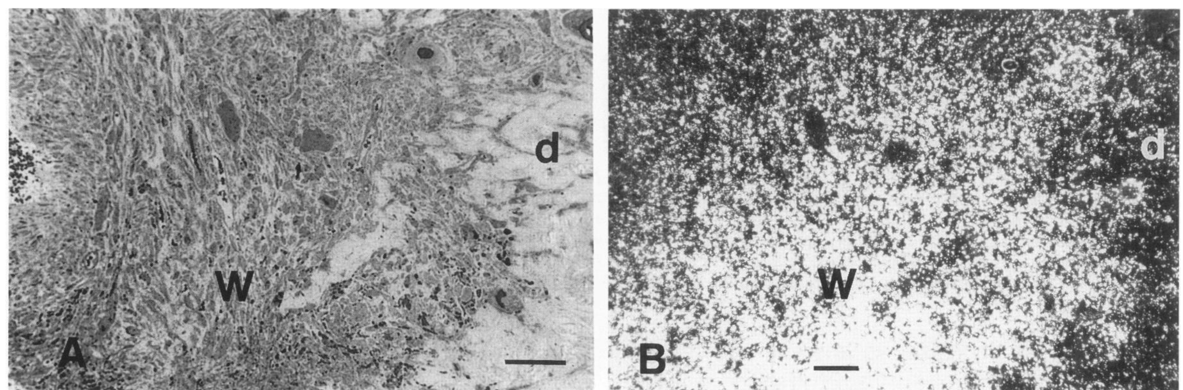


Figure 6. *Autoradiograph of a 7-day healing guinea pig punch wound that had been injected locally with [^{35}S]Na sulfate 6 and 3 hours before harvest. Tissue was fixed in paraformaldehyde-glutaraldehyde and Giemsa stained; 1-μ Epon sections were prepared for autoradiography.* **A**: *Bright-field image of healing wound (W) and adjacent normal dermis (d).* **B**: *Dark-field image of the same field showing intense labeling of wound stroma (W) as compared with normal dermis (d). Bars, 100 μ.*

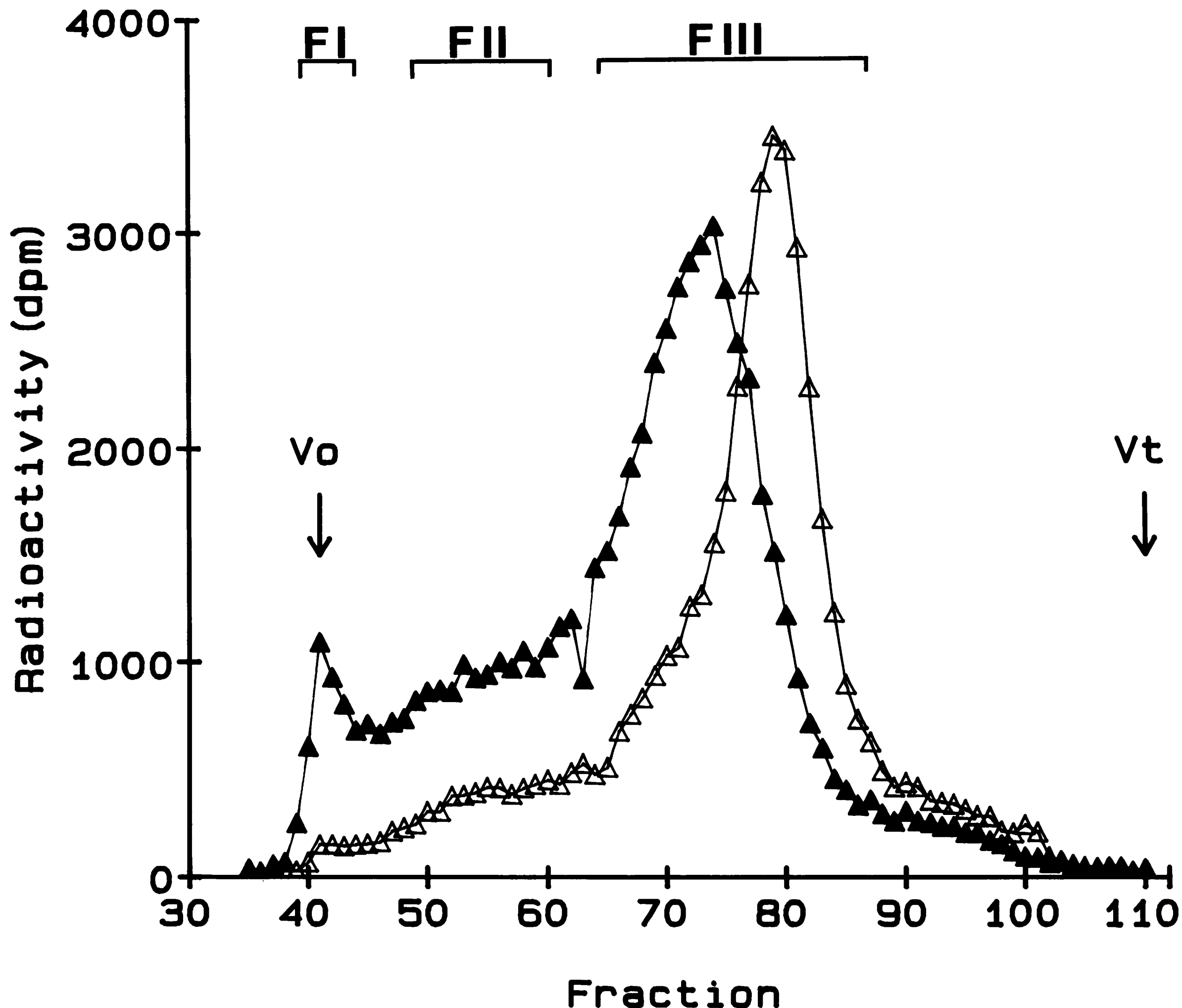


Figure 7. *Sepharose CL-4B chromatography of [^{35}S] Na sulfate-labeled proteoglycans isolated from normal dermis (open triangles) and from healing 7-day guinea pig skin wounds (filled triangles). 1×10^5 dpm of each sample were loaded onto a 0.9×100 cm Sepharose CL-4B column in 4 mol/l GuHCl buffer with 0.5% Triton X-100 in 0.1 mol/l TRIS-HCl, pH 7.0. V_o and V_t represent the void and the total volumes of the column, respectively. Fractions were pooled and designated FI, FII, and FIII, as indicated.*

dant decorin was demonstrated in normal skin by immunohistochemistry with the highly specific anti-PDS (decorin) antibody. In healing wounds, the major band isolated from fraction III migrated with an M_r ranging from 150,000 to 75,000 daltons (Figure 8, lane 1). Strong immunohistochemical staining (after chondroitin ABC lyase digestion) of healing wounds supports the view that this material is also decorin.

GAG Chains of the PG

The different species of normal skin and wound PG isolated by Sepharose CL-4B chromatography (Figure 7) were treated with alkaline borohydride to release the GAG chains. These then were digested with either chondroitin AC lyase or ABC lyase and chromatographed on Sepharose CL-6B columns. Wound fraction I contained almost entirely CS, fraction II mainly CS, and fraction III largely DS (Table 2). Similar proportions of fraction III of normal skin and healing skin wounds were digested with chondroitin AC lyase and ABC lyase (Table 2).

Sepharose CL-6B chromatography also permitted comparative sizing of GAG chains released from fractions II and III isolated from normal skin and healing wounds. (Insufficient amounts of labeled GAG chains were available for comparable studies of the fraction I.) The labeled chains (primarily CS) of fraction II from normal dermis eluted at Kav = 0.5 (M_r of ~ 20,000 daltons),[25] whereas those from healing wounds eluted at Kav = 0.35 (M_r of ~ 41,300 daltons; Figure 9A). The chains of fraction III of healing wounds (almost entirely DS, Kav of 0.44, M_r ~ 27,200 daltons) were approximately twice as large as those isolated from normal skin (Kav of 0.59, M_r ~ 12,400 daltons) (Figure 9B).

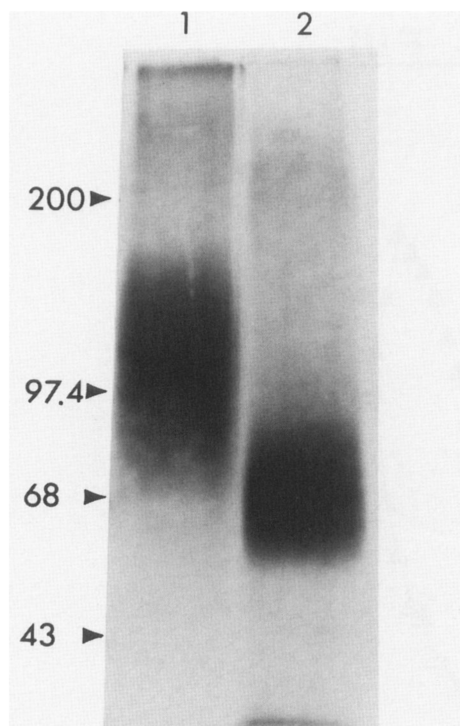


Figure 8. *SDS-PAGE of fraction III isolated by Sepharose CL-4B chromatography (Figure 5) from 7-day healing guinea pig skin wounds and from normal skin. Lane 1, healing wound; lane 2, normal control skin. Standard molecular weights are indicated (Mr × 10^{-3} daltons).*

Discussion

The studies reported here indicate that healing skin wounds and the stroma of several widely different types of carcinomas in guinea pigs and man share features of PG expression that distinguish them from the connective tissues of normal skin, breast, and colon. Thus healing skin wounds and the stroma of carcinomas of the skin, breast, bile duct, and colon all were characterized immunohistochemically by strong stromal staining for CS chains of CSPG and by little or no staining for decorin core protein. Lack of decorin staining in wounds and tumor stroma, however, was not attributable to the absence of immunologically reactive decorin core protein. Predigestion of tissue sections with chondroitin ABC lyase engendered strong decorin staining in both wounds and tumor stroma. Thus the decorin core protein is present in healing wounds and tumor stroma but without predigestion was apparently masked by GAG chains so as to be inaccessible to the anti-PDS antibody. In contrast, using an antibody directed against the N-terminal peptide of decorin, Adany et al[33] obtained staining of the stroma of one type of human tumor (colon carcinoma) without prior digestion with chondroitin ABC lyase. This discrepancy is most probably due to the different epitopes recognized by our different antibodies. Of note, these authors did not determine whether digestion with chondroitin ABC lyase increased carcinoma stromal staining. Taken together, our data demonstrate a common and distinct staining pattern of healing wounds and tumor stroma for both CSPG and decorin, adding yet another to the growing list of properties shared by healing wounds and tumor stroma.[1]

The immunohistochemical data presented is supported by the biochemical analytical data performed on the PGs synthesized by the normal dermis and wounds. A number of significant qualitative and quantitative differences distinguished the PG synthesized by 7-day healing guinea pig wounds from those of normal dermis. Our data may even understate the extent of these differences because wound tissue isolated by biopsy punch was unavoidably contaminated with at least some adjacent normal tissue. Wounds contained considerably more newly synthesized CSPG (found primarily in fraction I, and to a lesser extent in fraction II on Sepharose CL-4B chromatography; Figure 7) than normal adult skin. The DSPG composition of wound stroma also was altered. The major DSPG synthesized by both wounds and normal dermis most probably corresponds to decorin; however, in healing guinea pig skin wounds, the M_r of decorin was estimated to be ~70,000 daltons larger and more heterogeneous in size than that synthesized by normal skin (Figure 8). This difference in M_r is partly accounted for by the greater lengths of the wound DSPG GAG chains, which, on average, were more than twice as long in wounds (M_r, ~ 27,200 daltons) as in normal skin (M_r, ~ 12,400 daltons). The observed differences in GAG chain length, however, do not preclude other possible differences, such as an increased number of GAG chains per mole of core protein or differences in core protein structure not recognized by our antibodies. These other possibilities currently are being investigated. Of note, the DSPG isolated from normal skin and 7-day wounds had similar glucuronic and iduronic acid content, based on proportionate chondroitin AC lyase digestion (Table 2). Biochemical studies of tumor stroma PG comparable to those we have performed on healing wounds are in progress but are to some extent confounded by the fact that tumor cells themselves contribute to stromal PG, making it more difficult to assess the individual contributions made by fibroblasts and other cellular elements.

The source of the fibroblasts that constitute the granulation tissue of healing skin wounds has been disputed. At one time it was believed that wound fibroblasts arose from the emigration and local differentiation of circulating lymphocytes or monocytes.[34,35] The more modern and better documented view holds that most if not all wound fibroblasts arise from preexisting local tissue fibroblasts.[36] It is unclear, however, as to whether wound fibroblasts may be derived from any skin fibroblast or

Table 2. *Types of Glycosaminoglycan Chains Found in 7-day-old Guinea Pig Wounds and Normal Dermis, Expressed as % Digested with Chondroitin AC Lyase or ABC Lyase*

Column fraction	Chondroitin AC lyase		Chondroitin ABC lyase	
	Wound	Normal dermis	Wound	Normal dermis
Fraction I	5	ND	ND	ND
Fraction II	40	55	28	ND
Fraction III	86	83	7	14

Healing guinea pig skin wounds and normal dermis were labeled with [^{35}S-] Na sulfate, extracted, and chromatographed on Sepharose CL-4B to generate fractions I, II, and III as described in Methods. Glycosaminoglycan chains were released with alkaline borohydride, digested with either chondroitin AC lyase or ABC lyase, and fractionated on Sepharose CL-6B columns. The numbers represent the percentage of radioactive counts that remained *undigested* after chondroitin lyase digestion.

ND, Not determined.

from only a subpopulation of these cells. The immunohistochemical staining pattern of healing skin wounds for CSPG and decorin differed significantly from that of the majority of normal dermis, but did resemble that of discrete zones that together constitute only a minor fraction of the dermis; ie, thin rims of dermis disposed about blood vessels and adnexa, and focal deposits at the dermal–epidermal interface*. Therefore, if CSPG staining serves as a reliable marker that distinguishes different populations of fibroblasts, and if the fibroblasts in healing wounds maintain the CSPG synthetic pattern of their counterparts in normal skin, then it might be argued that the fibroblasts infiltrating healing skin wounds were derived from the minority population of fibroblasts thought to be responsible for the CSPG deposited in normal skin about blood vessels, adnexa, and at the dermal–epidermal interface. Indeed several authors have been led independently to just this conclusion on the basis of tritiated thymidine labeling studies.[37,38]

A similar issue arises with regard to the origin of the fibroblasts that generate tumor stroma. Because tumor stroma is CSPG positive and decorin negative with our antibody in the absence of prior chondroitin ABC lyase

*For the present discussion we have assumed that fibroblasts alone are responsible for synthesizing dermal PG. Epithelial and endothelial cells, pericytes, and smooth muscle cells also may synthesize PG, however, directly or indirectly by modifying fibroblast synthetic patterns. The role of these 'accessory' cells may be particularly important with regard to the CSPG-positive portions of normal dermis, which lie adjacent to epidermis, adnexa, and blood vessels.

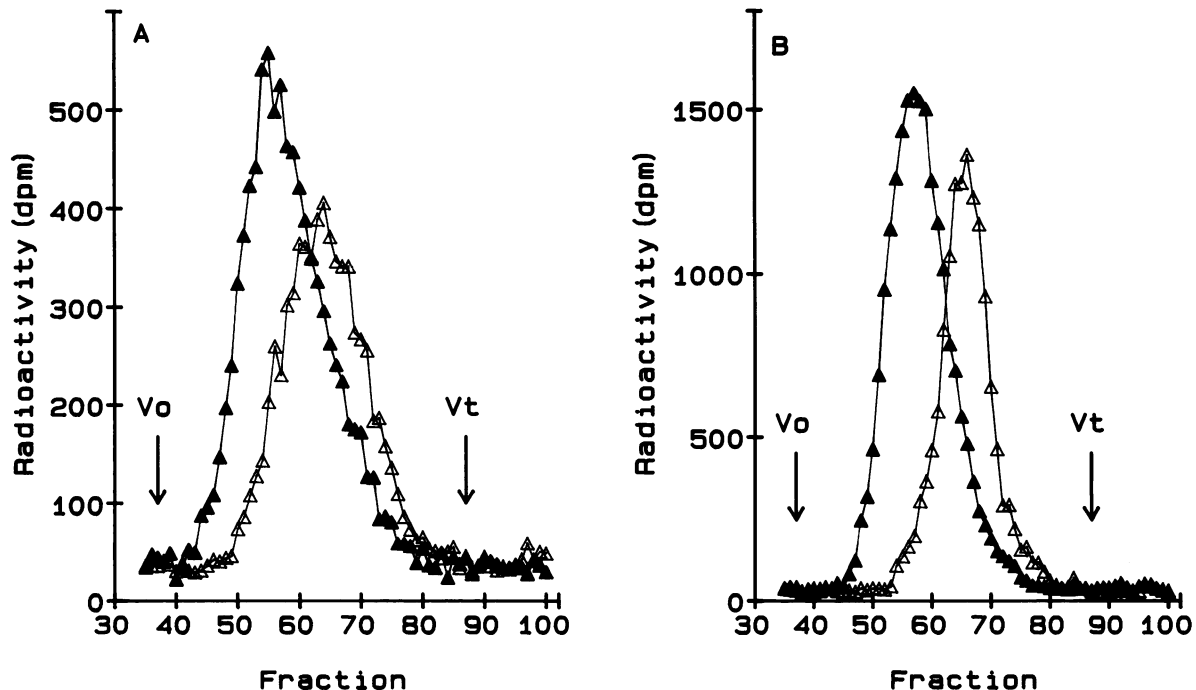


Figure 9. *Sepharose CL-6B chromatography of GAG chains isolated from wound and from normal control guinea pig skin PG by alkaline borohydride. Fraction II* (**A**) *and fraction III* (**B**) *fractions of wound (filled triangles) and normal skin (open triangles) extracts were isolated from Sepharose CL-4B columns (Figure 7), dialyzed, and precipitated with ethanol. The GAG chains from the samples were then released by alkaline-borohydride treatment and analyzed by Sepharose CL-6B column chromatography. V_o and V_t represent the void and total volumes of the column, respectively.*

digestion, it is tempting to postulate that tumor stroma fibroblasts are derived from normal connective tissue fibroblasts having a similar phenotype. In fact, connective tissues that gave a similar staining pattern to that of tumor stroma are found in select areas of normal skin, breast, and colon and are in every case the connective tissues most intimately associated with the epithelial cells from which each carcinoma arose (Table 1). The guinea pig carcinomas transplanted to the subcutis of syngeneic animals, however, also elicited stroma that was CSPG-positive/decorin-negative even though the tumor cells had been deposited amidst largely CSPG-negative/decorin-positive connective tissue. Finally tumor cells themselves synthesize PG that very likely contributed to the PG found in tumor stroma. For these reasons our data cannot establish that tumor stromal fibroblasts are derived from any specific fibroblast subpopulation of normal connective tissues.

There is, furthermore, an alternative and equally attractive explanation for our findings of altered PG synthesis in tumor stroma and wounds that does not require postulation of intrinsically different subpopulations of fibroblasts. This hypothesis holds that fibroblast synthetic patterns of PG are altered by growth factors and perhaps by other cytokines absent in normal connective tissues but present in inflammation and neoplasia. In fact, a number of different growth factors have been described in healing wounds or tumors, including platelet-derived growth factor (PDGF), epidermal growth factor, fibroblast growth factor, and transforming growth factors (TGF-α and β).[2–4,39] *In vitro,* TGF-β[40–42] and PDGF[43] stimulate several types of cultured cells to synthesize matrix CSPG and DSPG, and the molecular mass of the GAG chains synthesized is reported to be increased after TGF-β stimulation. Thus the altered PG synthesis observed in healing wounds and in tumor stroma may be attributable not to the preferential selection of a specialized subset of fibroblasts but to locally active growth factors that modify fibroblasts phenotype. Further studies will be necessary to determine whether one or some combination of both of these mechanisms best explains our immunohistochemical and biochemical findings.

The biologic significance of the altered pattern of PG synthesis in wounds and tumor stroma has yet to be determined. Studies of wounded endothelial cell cultures have demonstrated an increased accumulation of both CSPG and DSPG relative to HSPG.[44] Decorin binds to collagen,[11,12] and altered decorin synthesis might be expected to affect collagen fibrillogenesis and tensile strength.[13–15] Dermatan sulfate proteoglycan deposition has been correlated with collagen deposition in healing rabbit skin wounds,[45] and collagen affects DSPG synthesis and degradation.[46] Also an association has been found between external mechanical forces, collagen synthesis, and the synthesis of PG of different hydrodynamic sizes.[47] Thus explants obtained from proximal regions of bovine flexor tendon, which *in vivo* experience only tensile forces, have PG of relatively small hydrodynamic size and exhibit a high rate of collagen synthesis; conversely, explants of distal tendon, which *in vivo* experience frictional and compressive forces in addition to tensile forces, have a high content of larger proteoglycans and a lower rate of collagen synthesis.[47] Perhaps mechanical forces of these types contribute to the altered patterns of PG synthesis found in wound healing and tumor stroma generation. Recent studies have demonstrated that inhibition of PG synthesis by 4-methylumbelliferyl-β-D-xyloside reduced the accumulation of extracellular matrix in vascular smooth muscle cell cultures; the number of cytoskeletal filaments that contained α-actin also were reduced and cell proliferation and morphology were altered as well.[48] Thus PG may be expected to play important roles in the regulation of cell growth and differentiation, and the larger and more heterogeneous PG we have found in healing wounds may contribute importantly to the altered cell adhesion, migration, and proliferation associated with this process.

Acknowledgment

The authors thank Drs. T. N. Wight and H. Kresse for the gift of antibodies, Dr. J. Marcum for reading the manuscript and making helpful comments, Dr. M. G. Kinsella for helpful discussions, and E. J. Manseau and L. A. Freter for technical assistance.

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