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Original Article

Expression of Proteoglycans and Hyaluronan During Wound Healing[1]

OLLI OKSALA, TUULA SALO, RAIJA TAMMI, LARI HÄKKINEN, MARKKU JALKANEN, PIRJO INKI, and HANNU LARJAVA[2]

Departments of Periodontology (OO) and Oral Biology (LH), Institute of Dentistry, University of Turku, Turku, Finland; Department of Oral Surgery and Pathology (TS), University of Oulu, Oulu, Finland; Department of Anatomy (RT), University of Kuopio, Kuopio, Finland; Centre for Biotechnology (MJ,PI), Turku, Finland; and Department of Clinical Dental Sciences (HL), University of British Columbia, Vancouver, British Columbia, Canada.

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We investigated the expression of proteoglycans (PGs) and hyaluronan (HA) during healing of human mucosal wounds. Biopsy specimens of experimental wounds were taken 1, 3, and 7 days after wounding. Frozen sections were used for immunolocalization of CD44, syndecan-1, basement membrane-associated heparan sulfate proteoglycan (BM-HSPG), decorin, and biglycan. HA was localized in paraffin sections with a specific HA-binding probe. Epithelium showed first signs of migration on Day 1, more progressive migration on Day 3, and epithelial sheets confronted on Day 7. CD44 surrounded migrating keratinocytes at all stages of wound healing. In epithelium, CD44 and HA remarkably localized to the same region. Expression of syndecan-1 was switched from the suprabasal cell layer of unwounded epithelium to the basal cell layer of the migrating wound epithelium. BM-HSPG was absent under migrating keratinocytes. It started to reappear at the basement membrane zone on Day 7. The area under the wound epithelium containing newly synthesized collagen fibers first became positive for decorin on Day 7, whereas staining of biglycan was negative. Granulation tissue was also strongly positive for CD44 and hyaluronan. Our results indicate that migrating keratinocytes express both CD44 and syndecan-1 but not BM-HSPG. During differentiation of keratinocytes, expression of CD44 preceded that of syndecan-1. The results suggest that different HSPGs have multiple functions in keratinocyte migration and differentiation during reepithelialization. (*J Histochem Cytochem 43:125–135, 1995*)

KEY WORDS: Proteoglycans; Hyaluronan; Epithelium; Connective tissue; Wound healing.

Introduction

Proteoglycans (PGs) form a family of glycoconjugates associated with the extracellular matrix (ECM) and cell surfaces (for review see Kjellén and Lindahl, 1991; Uitto and Larjava, 1991; Ruoslahti, 1988). They are composed of a protein core substituted with one or more covalently linked glycosaminoglycans (GAGs). The nature of the GAG chain can be chondroitin–dermatan sulfate, –keratan sulfate, or –heparan sulfate in different PG species (Kjellén and Lindahl, 1991; Ruoslahti, 1988). Some core proteins can even carry both chondroitin and heparan sulfate, representing hybrid types of PGs. (Hardingham and Fosang, 1992; Jalkanen et al., 1992). The nature of the GAG chain can also vary in a tissue-specific fashion (Picker et al., 1989).

The amino acid sequences of many PGs have been recently obtained by cloning of their corresponding cDNAs (Carey et al., 1992; Kallunki and Tryggvason, 1992; Noonan et al., 1991; Fisher et al., 1989; Krusius and Ruoslahti, 1986), including syndecan-1, CD44, and perlecan, all of which are heparan sulfate-containing proteoglycans (HSPGs). These HSPGs localize specifically around the epithelial tissues and epithelial–mesenchymal boundaries. Syndecan-1 and CD44 are believed to function in binding of cells to the ECM (Jalkanen et al., 1992). Syndecan-1 also binds growth factors such as basic fibroblast growth factor (bFGF) (Bernfield and Sanderson, 1990). CD44 binds to hyaluronan (HA) and probably serves as a major HA receptor in many cell types (Aruffo et al., 1990; Miyake et al., 1990). Perlecan is the major PG component of the basement membranes (BMs). It is believed to function both as a structural element of the BM and in binding of growth factors (Noonan et al., 1991; Ruoslahti and Yamaguchi, 1991).

The major structurally known small dermatan sulfate proteoglycans (DSPGs) in oral soft connective tissue are highly homologous decorin and biglycan (Larjava et al., 1992; Bianco et al., 1990; Ruoslahti, 1988; Voss et al., 1986). Decorin has an affinity for Type I

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[2] Correspondence to: Dr. Hannu Larjava, Div. of Periodontics, Dept. of Clinical Dental Sciences, U. of British Columbia, 2199 Wesbrook Mall, Vancouver, BC, Canada V6T 1Z3.

collagen and regulates collagen fibril formation (Bianco et al., 1990; Brown and Vogel, 1989). The association of biglycan to Type I collagen has not been demonstrated, but it may bind to other fibrillar elements of the extracellular matrix (Häkkinen et al., 1993; Gallagher, 1989). Decorin is also believed to bind growth factors and to regulate cell growth (Yamaguchi et al., 1990).

The localization and function of PGs in various physiological processes are still largely unknown. For example, the distribution of various PGs during human wound healing is poorly understood (Weitzhandler and Bernfield, 1991). Wound healing involves several biological cell phenomena, such as cell migration, proliferation, BM regeneration, and the formation of granulation tissue. In this study we localized several PGs and HA during the initial healing of human mucosal wounds. We demonstrate that the expression of different PG species varies in a spatiotemporal fashion during wound healing.

Materials and Methods

Wounds. Experimental wounds were created in the palatal mucosa of healthy male and female volunteers. The protocol was approved by the Ethical Committee of the University of Turku, Finland. Incisions (~10 mm long, 1 mm wide, and 1–2 mm deep) were made about 5 mm from the gingival margin at the premolar area. Punch biopsy specimens were taken from wounds after 1, 3, and 7 days. Each specimen was cut in half; one half was mounted in Tissuetek and frozen in liquid nitrogen for use with fluorescence staining, and the other half was fixed with formalin and embedded in paraffin for use with routine histological techniques and immunoperoxidase staining. Frozen sections (6 μm) were cut on poly-D-lysine-coated slides and fixed in cold (−20°C) acetone for 5 min before use. Some sections were stained with hematoxylin and eosin to demonstrate the basic histology of wound healing, and with phosphotunstic acid–hematoxylin (PTAH) to demonstrate the presence of fibrin.

Antibodies. PGs were localized with the specific antibodies listed in Table 1. Biglycan antipeptide serum recognizes a specific peptide sequence at the amino terminus of the molecule (Fisher et al., 1989). Monoclonal antibody (MAb) Hermes-3 against CD44 recognizes the core protein of all known CD44 species (Goldstein et al., 1989). Decorin antibody specifically recognizes the amino terminal peptide sequence of the core protein (Krusius and Ruoslahti, 1986). HSPG in the basement membrane area was recognized with a specific antibody against core protein of BM-HSPG (Kemeny et al., 1988). Syndecan-1 was localized with a polyclonal antipeptide antibody that specifically binds to syndecan-1 core protein in immunoblots (Inki et al., 1994). HA was recognized with a specific probe (HABC) prepared from the hyaluronan-binding region of cartilage PG (Wang et al., 1992; Ripellino et al., 1985). Rhodamine-conjugated secondary antibodies were affinity-purified, one against rabbit and the other against mouse IgGs (Boehringer Mannheim; Indianapolis, IN).

Table 1. *Antibodies used in the present study*

Antigen	Antibody	Reference
Biglycan	MAb LF-51	Fisher et al., 1989
CD44	MAb Hermes-3	Jalkanen et al., 1987
Decorin	Polyclonal anti-peptide antibody	Krusius and Ruoslahti, 1986
BM-HSPG	MAb 458, clone 7E12	Chemicon International; Temecula, CA
Syndecan-1	Polyclonal anti-peptide antibody	Inki et al., 1994

Immunolocalization. To remove chondroitin sulfate chains in PG core proteins, the frozen sections were treated with 1.25 U/ml of chondroitinase ABC from *Proteus vulgaris* (Seikagaku Kogyo; Tokyo, Japan) in buffer [0.5 M Tris, 0.05 M sodium acetate, 0.01% bovine serum albumin (BSA; Sigma, St Louis, MO), pH 7.2], at 37°C for 10 min. After five rinses in PBS, nonspecific staining was blocked by incubating slides for 30 min in PBS containing 0.1% BSA (0.1% BSA–PBS).

The primary antibodies (Table 1) were diluted in 0.1% BSA–PBS and used at a concentration that had been tested to give the best immunoreaction compared to background staining. After 60 min, sections were washed four times in 0.1% BSA–PBS, 15 min each. Rhodamine-conjugated secondary antibodies (1:50, in 0.1% BSA–PBS) were applied on the sections for 60 min. After rinsing, the sections were washed twice in distilled water for 10 min and air-dried at room temperature (RT). The sections were then mounted with cyanoacrylate adhesive (Extra Super Glue; Chemo-Tech Chemical, Taiwan). Control stainings were carried out as described above with non-immune rabbit serum (1:100) as the primary antibody and rhodamine-conjugated anti-rabbit IgG as the secondary antibody.

Immunofluorescence was analyzed with a Leitz Aristoplan research microscope equipped with UV light as epifluorescence. Stainings were photographed on Kodak Tri-X Pan 400 film using Leiz Aristoplan photographing automat.

Localization of CD44 and HA in parallel paraffin-embedded sections was performed as described earlier (Wang et al., 1992). Briefly, for HA staining, sections were incubated in 1% BSA for 30 min and then with biotinylated HABC (5 μg/ml) overnight at 4°C. Sections were then washed and incubated in avidin–biotin–peroxidase 1:200 (Vector Laboratories; Irvine, CA) for 1 hr and thereafter stained in 0.05% 3,3'-diaminobenzidine (Sigma) and 0.03% H_2O_2 in buffer until the color developed. For immunostaining of CD44, sections were incubated with 1% BSA for 30 min. Thereafter, MAb Hermes-3 (1:300) was incubated on slides overnight at 4°C. Endogenous peroxidase activity was blocked by incubating slides with 0.03% H_2O_2 in anhydrous methanol for 3 min. Sections were incubated with biotinylated anti-mouse secondary antibody (1:100; Vector) and avidin–biotin–peroxidase (1:200) for 1 hr at RT. After rinsing, CD44 was visualized by the 3,3'-diaminobenzidine reaction as described above. Sections were mounted with Permount and analyzed by light microscopy.

Results

Morphology of Healing Mucosal Wounds

Routine histochemical stainings [hematoxylin and eosin (HE) staining, fibrin staining by PTAH] were used to demonstrate the migration pathway of the epithelium and the formation of granulation tissue (Figures 1D–1F). In 1-day-old wounds, clot was easily detectable in HE stainings (Figure 1D). At the wound margin, the epithelial sheet showed signs of initial movement into the wound space. However, the migration was still limited. Three days after wounding, the epithelial sheets from the wound margin had migrated about 250 μm into the wound space (Figure 1E). The migrating sheets appeared to progress through the clot rather than below it. In 7-day-old wounds, the epithelial sheets had confronted and closed the wound gap that was still detectable by HE and fibrin stainings (Figures 1F and 5D). Some fibrin was still seen in the granulation tissue in which collagen fibers first started to appear (Figure 5D). Based on the colors of the PTAH staining (old collagen red and new collagen pink), we found that collagen was first deposited under the newly formed epithelium.

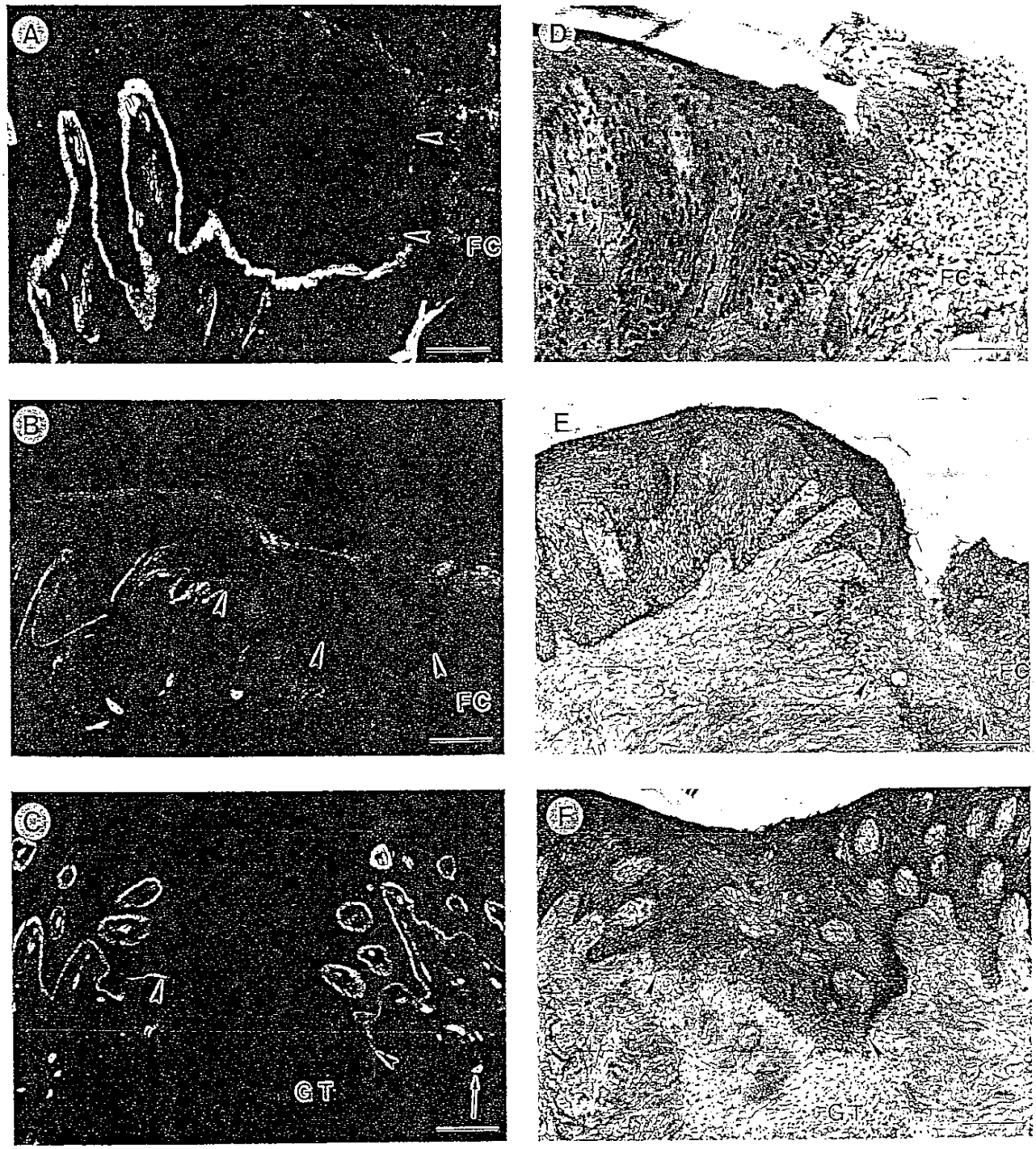


Figure 1. Expression of BM-HSPG in (A) 1-day-old, (B) 3-day-old, and (C) 7-day-old wounds. Arrowheads mark the wound margins (A,C), and migrating epibolus (B). A blood vessel positive for BM-HSPG is marked with arrow. (D–F) Corresponding hematoxylin and eosin stainings of the wounds. FC, fibrin clot; GT, granulation tissue. Bars: A,D = 20 μm; B,C,E,F = 50 μm.

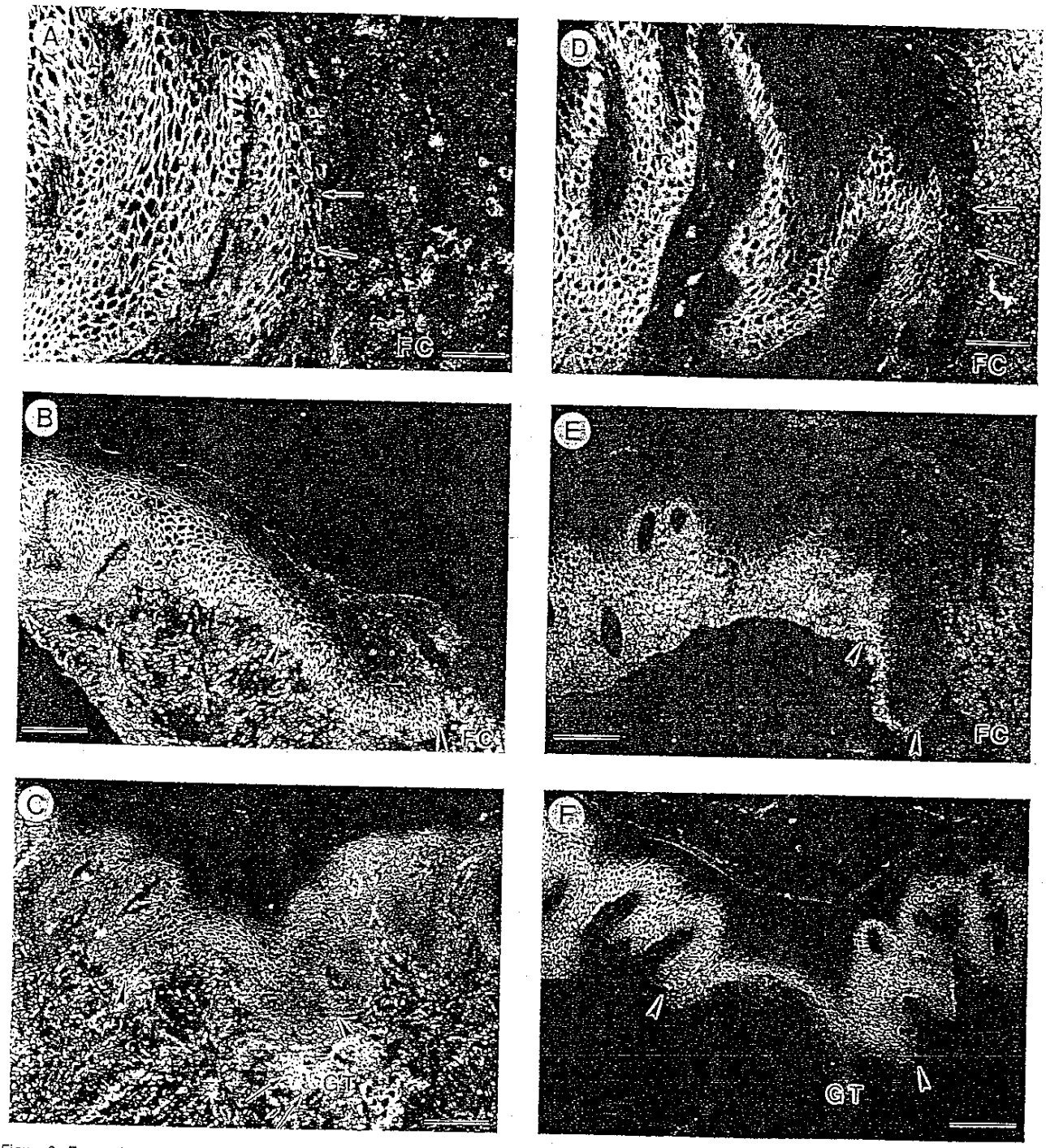
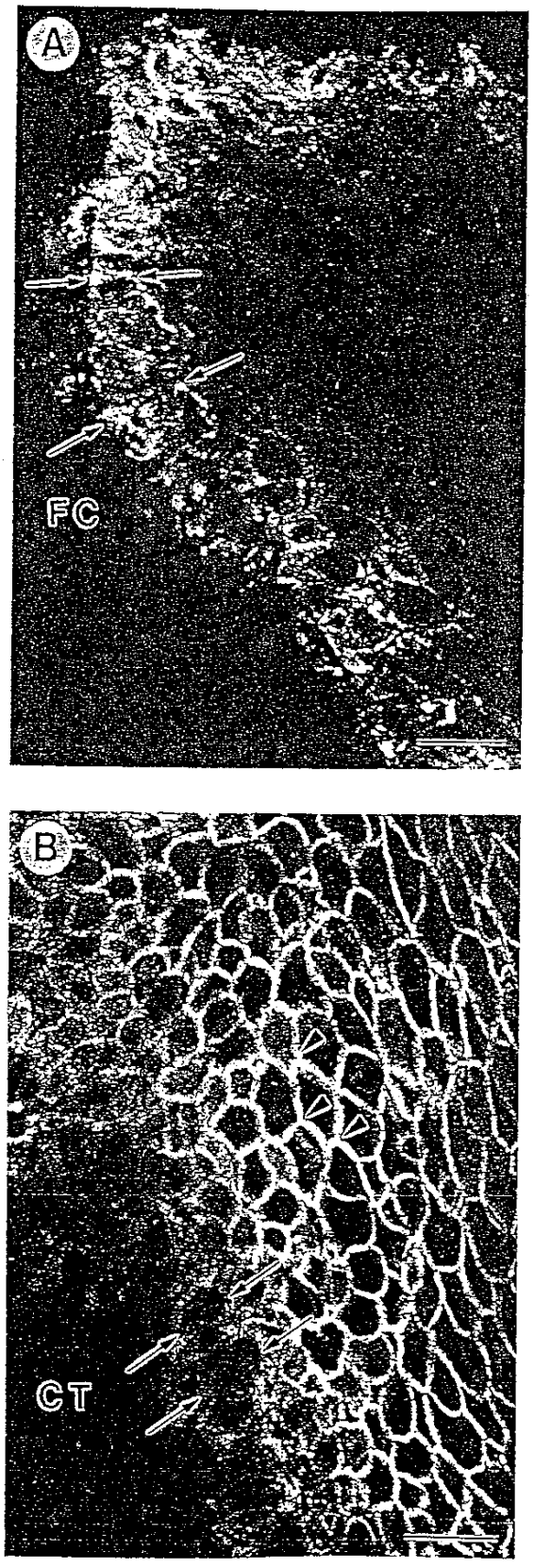


Figure 2. Expression of (A–C) CD44 and (D–F) syndecan-1 in 1-day-old (A,D), 3-day-old (B,E), and 7-day-old wounds (C,F). Arrows mark the wound margin on Day 1; arrowheads mark the migrating epibolus on Day 3, and the wound margins on Day 7, respectively. FC, fibrin clot; GT, granulation tissue; Bars: A,D = 20 μm; B,C,E,F, = 50 μm.

Immunolocalization of BM-HSPG, CD44, and Syndecan-1 in Healing Wounds

HSPG was specifically localized at the BM zone of the non-wounded mucosal tissue (Figures 1A–1C). It was also localized in the walls of some blood vessels. No marked changes were seen in the staining at the wound margin in 1-day-old wounds (Figure 1A). BM-HSPG was, however, absent under the migrating epibolus in 3-day-old wounds (Figure 1B). After complete epithelialization of 7-day-old wounds (Figure 1C), first signs of HSPG expression at



the regenerating BM zone were evident but were visible only at higher magnification (not shown). However, this staining was weak and discontinuous. Granulation tissue blood vessels appeared negative for BM-HSPG expression (Figure 1C).

CD44 was immunolocalized in mucosal wounds by Hermes-3, an MAb that recognizes epitopes in the constant part of the CD44 molecule (Figures 2A–2C). It allows the localization of all epithelium-, leucocyte-, and fibroblast-specific CD44 species (Picker et al., 1989). In non-wounded mucosa, CD44 was localized in all layers of the stratifying epithelium except for stratum corneum, and throughout the connective tissue when MAb Hermes-3 was used. In the epithelium, CD44 decorated the surroundings of the stratifying keratinocytes. In 1-day-old wounds, the first epithelial cells that had started lateral migration were rather weakly CD44-positive (Figure 2A). Some blood cells inside the fibrin clot were also Hermes-3-positive. Migrating epithelial cells in 3-day-old wounds were CD44-positive in a fashion similar to the normal mucosal epithelium (Figure 2B). In 7-day-old wounds, CD44 localization around the keratinocytes resembled that of the normal mucosa, except that a few more upper cell layers were still negative (Figure 2C). Newly formed granulation tissue strongly expressed the Hermes-3 antigen (Figure 2C). In 1-day-old wounds, the keratinocytes at the wound margin were almost negative for syndecan-1 expression (Figure 2D). In the migrating epibolus of 3-day-old wounds, only the most basal cell layer was syndecan-1 positive, whereas suprabasal cell layers were negative (Figure 2E). After complete reepithelialization of 7-day-old wounds, syndecan-1 was still absent in the various upper cell layers of the confronted epithelium (Figures 2F and 5C). The granulation tissue was negative for syndecan-1 expression (Figure 2F). Higher magnification revealed that syndecan-1 in nonaffected oral mucosa was localized only in the epithelium, where it surrounded the keratinocytes in several stratifying cell layers (Figure 3B). However, the basal cell layer was weakly stained by the syndecan-1 antibody (Figure 3B). During migration, only the most basal cells of the epibolus were syndecan-1 positive (Figure 3A).

Localization of Small DSPGs (Decorin and Biglycan) in Healing Wounds

Decorin was abundant in the connective tissue of non-affected oral mucosa, as shown previously (Häkkinen et al., 1993). No decorin was found in the wound area on Day 1 (Figure 4A) and Day 3 (Figure 4B). First signs of decorin deposition were seen under confronted epithelium on Day 7 (Figures 4C and 5B). Biglycan was distributed throughout the mucosal connective tissue of non-affected sites but was absent from the wound site at all time points studied, Day 1 (Figure 4D), Day 3 (Figure 4E), and Day 7 (Figure 4F).

Figure 3. Expression of syndecan-1 in (A) migrating epithelium and (B) in epithelium of non-wounded mucosa. Arrowheads mark positive staining of syndecan-1 in stratifying keratinocytes (B). Arrows mark syndecan-1-negative basal cells in non-wounded area but positive in migrating epithelium of a 3-day-old wound. CT, connective tissue; FC, fibrin clot. Bar = 10 μm.

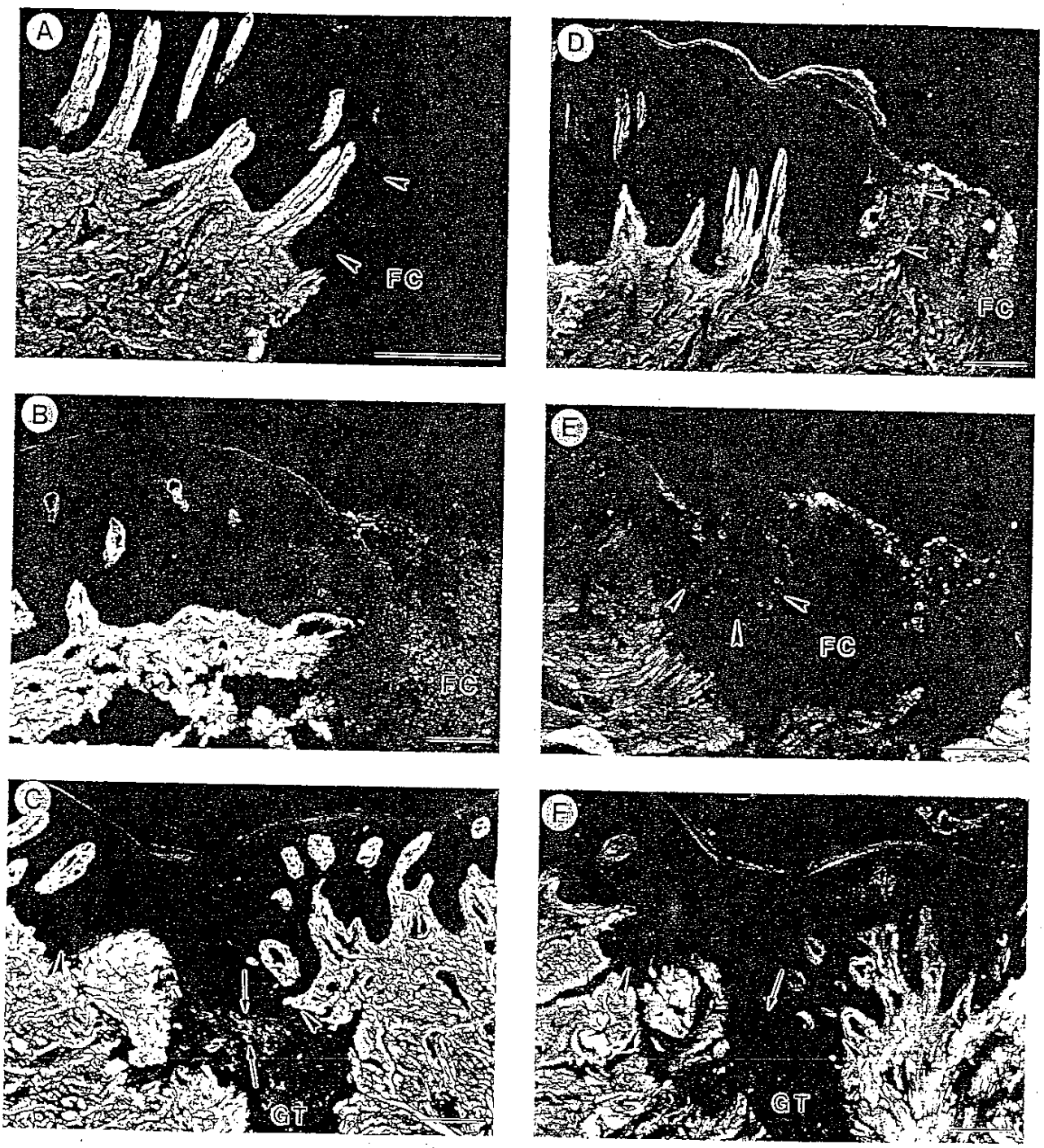


Figure 4. Immunolocalization of (A–C) decorin and (D–F) biglycan in 1-day-old (A,D), 3-day-old (B,E), and 7-day-old wounds (C,F). Arrowheads mark the wound margins (A,D,C,F) and migrating epibolus (E). Arrows mark the subepithelial granulation tissue strongly positive for decorin (C) but negative for biglycan (F). (B,D) Some background staining is visible in FC. FC, fibrin clot; GT, granulation tissue. Bar = 50 μm.

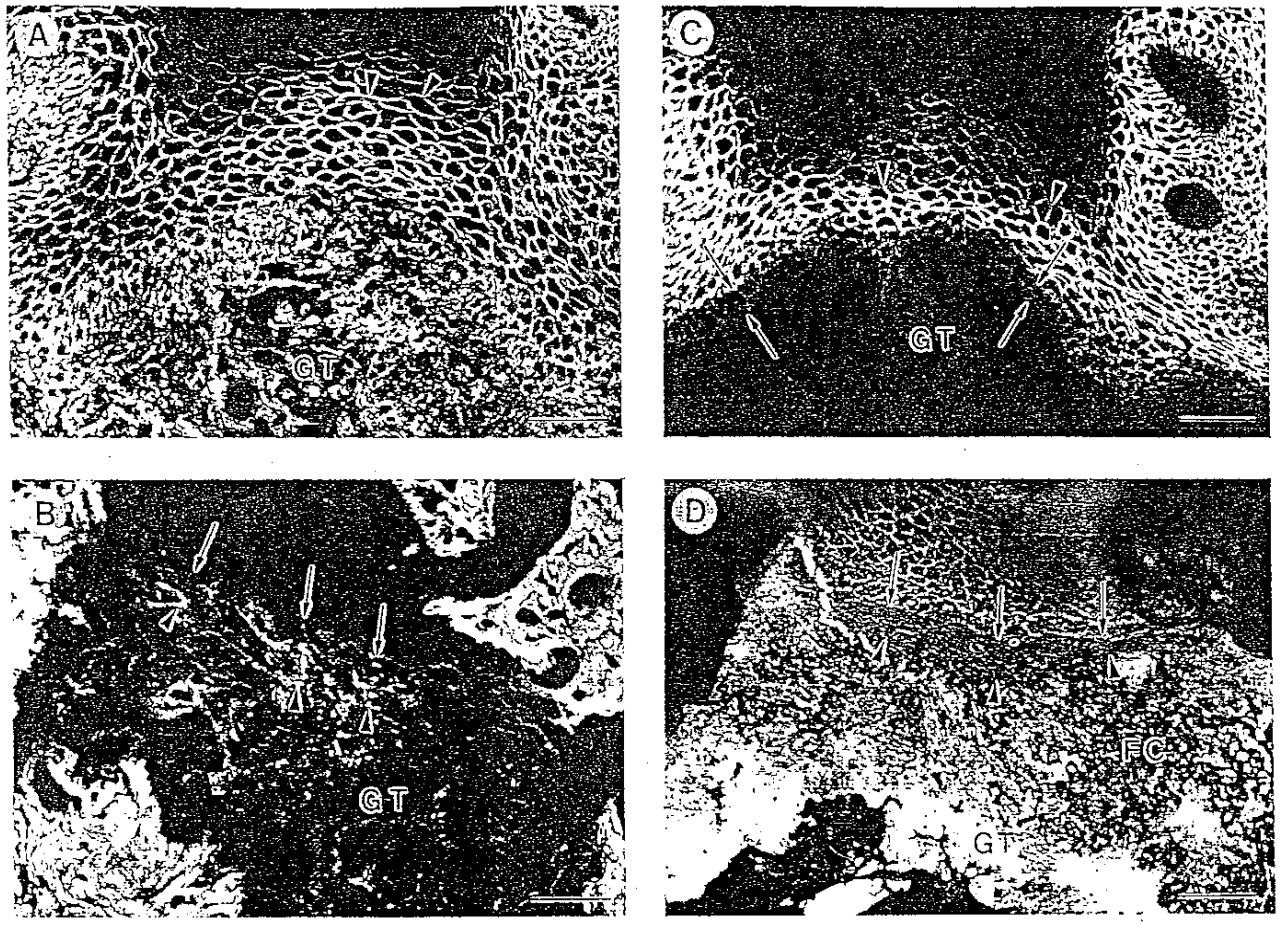


Figure 5. Immunolocalization of (A) CD44, (C) syndecan-1, and (B) decorin in 7-day-old wounds. CD44 and syndecan-1 localize in cell–cell contacts of stratifying keratinocytes (A,C, arrowheads). Basal cell layer is negative for syndecan-1 (C, arrows). Decorin appears first in the subepithelial granulation tissue (between arrows and arrowheads in B), at the same site with first collagen fibers (between arrows and arrowheads in fibrin staining, D), in parallel sections of the same specimen. GT, granulation tissue; FC, fibrin clot. Bar = 20 μm.

Localization of PGs in Granulation Tissue and Confronted Epithelium

Expression of different PGs in granulation tissue and confronted epithelium was then compared with higher magnification (Figure 5). CD44 was well expressed at the granulation tissue where syndecan-1 was absent (Figures 5A and 5C). In epithelium, the localization resembled that of non-affected areas. However, in uppermost cell layers of the epithelium, syndecan-1 in particular was still negative. The first signs of decorin expression could be seen in the same area as the first collagen bundles (Figures 5B and 5D), whereas no signs of biglycan expression were evident (Figure 4F).

Localization of HA In Healing Wounds

Because CD44 is considered an important receptor for HA in many cell types (Aruffo et al., 1990; Miyake et al., 1990; Picker et al., 1989), we tried to determine whether CD44 localizes with HA to the same area of healing mucosal wounds, using parallel paraffin sections from the same specimen (Figure 6). HA and CD44 were localized to the same region of the epithelium (around mucosal keratinocytes) in all stages of wound healing (compare Figure 6A–6C with Figures 6D–6F). In normal connective tissue and granulation tissue, however, CD44 staining was restricted in cells, whereas HA was diffusely distributed throughout the tissue.

Hyaluronan was strongly present around the epithelial keratinocytes in all cell layers except for the stratum corneum of the non-wounded epithelium (Figure 6), as has been reported earlier (Tammi et al., 1990). Connective tissue matrix was also diffusely stained for HA. In 1-day-old wounds, accumulation of HA was seen at the wound margin (Figure 6D). Migrating keratinocytes of the epibolus were HA-positive, but the uppermost layers were HA-negative (Figure 6E). In 7-day-old wounds, the confronted epithelium was almost normally stained for HA, except for the less intensely stained epithelial depression in the middle of the wound (Figure 6F). The newly formed granulation tissue was strongly positive for HA (Figure 6F).

Discussion

In this study we localized three epithelial HSPGs and two soft connective tissue DSPGs in full-thickness mucosal wounds of human

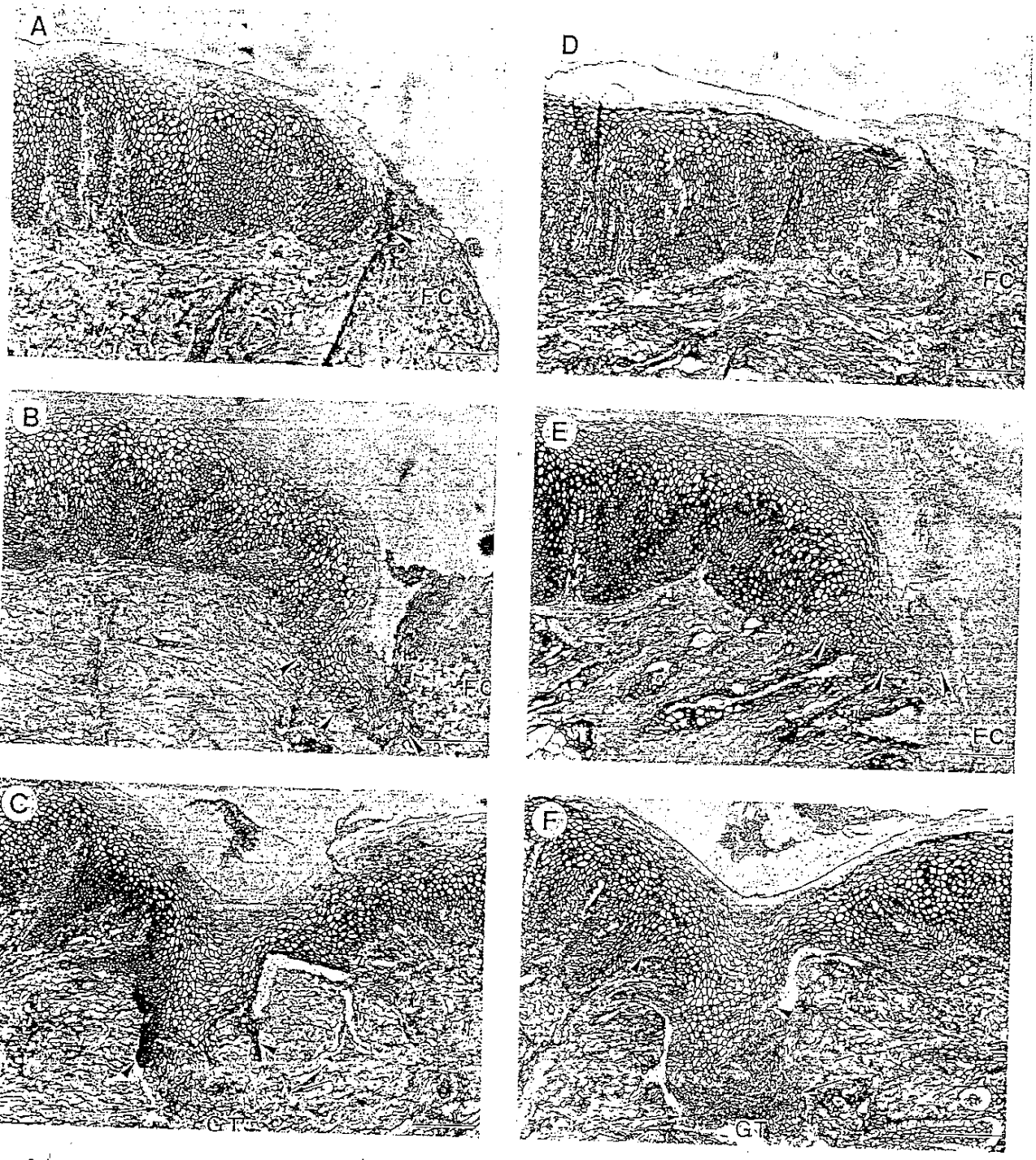


Figure 6. Immunolocalization of (A–C) CD44 and (D–F) hyaluronan in 1-day-old (A,D), 3-day-old (B,E), and 7-day-old wounds (C,F). Wound margins and migrating epibolus are marked by arrowheads. Arrows mark granulation tissue (GT). FC, fibrin clot. Bar = 50 μm.

subjects. We demonstrate that syndecan-1, CD44, and BM-HSPG are differentially expressed during keratinocyte migration. Furthermore, during granulation tissue formation decorin appears in the tissue along with the first collagen fibers, whereas the expression of biglycan is still undetectable.

HSPGs are found in the extracellular matrix and on cell surfaces (Gallagher et al., 1986). They regulate cell–cell and cell–matrix interactions and also bind growth factors (Ruoslahti and Yamaguchi, 1991). In this study we have shown that CD44 and HA localize at the same region during healing of human mucosal wounds. In wounds, expression of HA is increased (Weigel et al., 1989) and may serve as a ligand for cell adhesion. It is possible that epithelial CD44 has a role in recognizing HA in keratinocyte–matrix interactions and therefore has an important role in reepithelialization during wound healing. Although an epithelial variant of CD44 has been reported to bind HA only weakly in vitro (Stamenkovic et al., 1991), it is still possible that the HA-binding site of epithelial CD44 is expressed by a proper protein folding. Even by Day 1, the lateral sides of a wound bed had become positive for HA. This suggests that HA is first deposited at the areas to which keratinocytes are expected to migrate and that this migration may then be regulated via cell surface-associated CD44. Binding of HA is probably not the only function of CD44 in keratinocytes. CD44 is a multifunctional cell surface PG that binds high endothelial venule (HEV) ligand (Jalkanen et al., 1987), HA, fibronectin, laminin, and collagens (Wayner and Carter, 1987). These ECM components are exposed during wound healing and are well-known adhesion ligands for keratinocytes (Stenn et al., 1983; Terranova et al., 1980; Murray et al., 1979). Both CD44 and HA were also strongly expressed in forming granulation tissue. Therefore, it is possible that CD44 functions as an HA-binding receptor in granulation tissue cells, such as macrophages and fibroblasts.

Syndecan-1 expression in wound healing was restricted in basal keratinocytes of the migrating epithelial sheet. Basal cells of the normal mucosa, however, showed relatively weak expression of syndecan-1. Because of its localization over the entire surface of keratinocytes, syndecan-1 has been suggested to function as a cell–cell adhesion molecule in the epithelium (Hayashi et al., 1987). During wound healing, keratinocytes are exposed to ECM molecules, many of which are known ligands for syndecan-1 (Salmivirta et al., 1991; Kiefer et al., 1990; Sun et al., 1989; Saunders and Bernfield, 1987; Koda et al., 1985). We found that the basal cell layer of migrating keratinocytes was positive for syndecan-1. Therefore, it is possible that, during wound healing, syndecan-1 acts as an ECM-binding receptor. Syndecan-1 staining in migrating keratinocytes resembles that of integrin receptors (αv and $\alpha 5\beta 1$) (Larjava et al., 1993). We can further hypothesize that syndecan-1 and integrins may function as co-receptors in matrix binding in wound keratinocytes. In cultured cells, this kind of cooperation has been demonstrated (Woods et al., 1985). In addition, syndecan-1 may also function in growth factor recognition during wound healing, since growth factor levels are known to be elevated at wound areas (Werner et al., 1992). Our finding that syndecan-1 is rather weakly expressed in the epibolus agrees with a recent report concerning mouse epidermal healing (Elenius et al., 1991). Induction of syndecan-1 in proliferating wound margins was not so striking as that seen in the mouse model (Elenius et al., 1991). Our study deals, however, with a model in which glands or hair follicles do not contribute to the healing process. We also suggest that syndecan-1 has the ability to change its function from cell–cell to cell–ECM interaction, and vice versa. Whether or not this happens via the different syndecan isoforms (Sanderson and Bernfield, 1988) remains to be determined.

BM-associated HSPGs are a diverse group of PGs. Perlecan, a large 400–500 KD BM-associated HSPG, has been recently completely sequenced (Kallunki and Tryggvason, 1992; Noonan et al., 1991). The antibody used in this experiment recognized an HSPG with a molecular weight of 210 KD (Kemeny et al., 1988). Even though the smaller BM-HSPGs have been suggested to be proteolytic fragments of a large BM-HSPG (Klein et al., 1988; Ledbetter et al., 1985), it remains unknown whether the antibody we used recognized the perlecan fragments or HSPGs of another origin. BM-HSPG was absent under migrating epithelial cells. It appears, therefore, that it follows the sequence of deposition of other classical BM components (laminin, Type IV collagen) and Type VII collagen (Larjava et al., 1993) during wound healing. Because BM-HSPG was not expressed by migrating keratinocytes, we propose that it is not used by the cells during migration but rather is needed for regeneration of the biological functions of BM. It was interesting that only a few endothelial BMs in normal mucosa showed positive staining for perlecan. In addition, the new capillaries in the granulation tissue were completely negative. However, in a previous study we have shown that other classical BM components, such as laminin and Type IV collagen, are present in forming blood vessels of this area (Larjava et al., 1993). It can be speculated that BM-HSPG expression in granulation tissue is low, allowing greater penetration of serum components into the regenerating tissue.

The first signs of decorin expression were seen under the confronted epithelium at the same area in which the first collagen bundles were deposited. Because decorin has a high affinity for Type I collagen (Bianco et al., 1990), it is most likely that decorin regulates collagen deposition in healing wounds. Expression of soft connective tissue PGs is regulated by transforming growth factor β (TGFβ), which stimulates the expression of biglycan but downregulates that of decorin in vitro (Kähäri et al., 1991). During wound healing, we did not note elevated expression of biglycan over decorin in granulation tissue. In fact, biglycan was still absent in 7-day-old granulation tissue. It is conceivable, therefore, that the expression of PGs in wounds is regulated by a number of cytokines in a concerted manner.

In summary, we have demonstrated for the first time in humans the localization of major epithelial and connective tissue PGs during wound healing. CD44 may function as an HA receptor in wound healing, and syndecan-1 in recognition of ECMs. Decorin may regulate connective tissue regeneration via collagen fibril formation. It is obvious that all these PGs have a specific spatiotemporal distribution during tissue regeneration, indicating their importance in normal epithelial and connective tissue biology.

Acknowledgments

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