Induced Expression of Syndecan in Healing Wounds

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Abstract. We have studied the expression of an integral cell surface proteoglycan, syndecan, during the healing of cutaneous wounds, using immunohistochemical and in situ hybridization methods. In normal mouse skin, both syndecan antigen and mRNA were found to be expressed exclusively by epidermal and hair follicle cells. After incision and subsequent suturing, remarkably increased amounts of syndecan on the cell surfaces of migrating and proliferating epidermal cells and on hair follicle cells adjacent to wound margins were noted. This increased syndecan expression was shown to be a consequence of greater amounts of syndecan mRNA. Induction was observed already 1 d after wounding, was most significant at the time of intense cell proliferation, and was still observable 14 d after incision. The migrating cells of the leading edge of the epithelium also showed enhanced syndecan expression, although clearly less than that seen in the proliferating epithelium. The merging epithelial cells at the site of incision showed little or no syndecan expression; increased syndecan expression, however, was detected during later epithelial stratification.

When wounds were left unsutured, in situ hybridization experiments also revealed scattered syndecan-positive signals in the granulation tissue near the migrating epidermal sheet. By immunohistochemical analysis, positive staining in granulation tissue was observed around vascular endothelial cells in a subpopulation of growing capillaries. Induction of syndecan in granulation tissue both at the protein and mRNA levels was temporally and spatially highly restricted. Granulation tissue, which formed in viscose cellulose sponge cylinders placed under the skin of rats, was also found to produce 3.4 and 2.6 kb mRNA species of syndecan similar to that observed in the normal murine mammary epithelial cell line, NMuMG. These results suggest that syndecan may have a unique and important role as a cell adhesion and a growth factor–binding molecule not only during embryogenesis but also during tissue regeneration in mature tissues.

BOTH migrating and proliferating cells interact with surrounding matrices. Such interactions are mediated by cell surface receptors, can provide positional information for cells and can initiate intracellular signal transmission (see Damsky and Bernfield, 1990). Matrix receptors allow detaching and rebinding again of cells to extracellular matrix (ECM)[1] molecules in a manner that is regarded as highly coordinated. It is also obvious that more than one single molecule facilitates these multiple tasks, as the number of matrix molecules is abundant. Some well known cell surface molecules participating in cell–cell and cell–matrix interactions include integrins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Abelda and Buck, 1990), CAMs of the IgG superfamily (Edelman, 1988), cadherins (Takeichi, 1990) and cell surface proteoglycans (Ruoslahti, 1989).

One characterized cell surface proteoglycan is syndecan, which has a 33-kD core protein with highly conserved transmembrane and cytoplasmic domains (Saunders et al., 1989; Kiefer et al., 1990; Mali et al., 1990). The ectodomain of syndecan contains both heparan sulfate and chondroitin sulfate (Rapraeger et al., 1985; Elenius et al., 1990) and binds selectively to fibrillar collagens (Koda et al., 1985), to heparin binding domains of fibronectin (Saunders and Bernfield, 1988), to thrombospondin (Sun et al., 1989) but neither to vitronectin nor to laminin (Elenius et al., 1990). Polymorphic forms of syndecan exist in different tissues as a result of differential glycosylation (Sanderson and Bernfield, 1988), and the resulting variations can also elicit alterations in matrix recognition. This has been observed recently for tooth mesenchyme-derived syndecan, which contains only heparan sulfate and which selectively binds tenascin (Salmivirta et al., 1991). On the other hand, ligand binding can promote the close interaction of syndecan with actin-rich cytoskeleton (Rapraeger et al., 1986); however, cells can

1. *Abbreviations used in this paper*: ABC, avidin–biotin complex; bFGF, basic fibroblast growth factor; PAP, peroxidase–antiperoxidase; TGFβ, transforming growth factor.

also detach themselves from this interaction by proteolytic cleavage of the core protein, resulting in the shedding of the matrix binding ectodomain from the cell surfaces (Jalkanen et al., 1987). By such associations, syndecan can participate in the regulation of cell shape, a function essential to the maintenance of cell morphology and phenotype. This hypothesis has been recently supported by the observation that the loss of hormonally-regulated epithelial phenotype correlates to the suppression of syndecan gene in S115 mouse mammary tumor cells (Leppä et al., 1991).

Syndecan also behaves like a cell–cell adhesion molecule. It is expressed around stratified epithelial cells of several tissues (Hayashi et al., 1987); and its staining patterns in morphologically active tissues, including several differentiating mesenchymes (Thesleff et al., 1988; Vainio et al., 1989*a*,*b*; Solursh et al., 1990), suggest a role in cell–cell adhesion. Syndecan also binds some growth factors, e.g., basic fibroblast growth factor (bFGF) (Kiefer et al., 1990). It may also, therefore, participate in the modulation of growth factor effect on various cells (Ruoslahti and Yamaguchi, 1991). The spatial and temporal expression of syndecan during development, participation in the maintenance of cell shape as well as its interactions with extracellular effector molecules indicate that syndecan may take part in the regulation of diverse biological phenomena such as proliferation, differentiation and morphogenesis. Besides development, these biological processes are also utilized during tissue regeneration of which wound healing is a good example. We have studied, in this paper, regulation of syndecan expression during healing of cutaneous wounds. These studies revealed an enhanced expression of syndecan in proliferating and migrating epithelial cells of epidermis and hair follicles but also, surprisingly, limited expression of syndecan on the surface of vascular endothelial cells of granulation tissue. Therefore, syndecan (or its variants) may also participate in the regulation of endothelial differentiation.

Materials and Methods

Cell Culture

NMuMG mouse mammary epithelial cells and 3T3 (NIH) mouse fibroblasts were cultured in bicarbonate-buffered DMEM (Gibco Laboratories-BRL, Paisley, UK) containing 10% FCS (Gibco Laboratories-BRL) and antibiotics, as previously described (Elenius et al., 1990). For RNA isolation, cells were grown to 70–80% confluency, washed with ice-cold PBS and solubilized in 4 M GIT buffer (4 M guanidine isothiocyanate, 5 mM sodium citrate (pH 7.0), 0.1 M β-mercaptoethanol, and 0.5% *N*-laurylsarcosine).

Preparation of Tissues

For immunohistochemical and in situ hybridization experiments, wound tissue was obtained from 3-mo-old male Balb/c mice. Animals were anesthetized with ether. After shaving, a 6-mm-long full-thickness incision was made in the dorsal midline in the caudal part of the back skin. Wounds were either sutured immediately after incision or left uncovered. Surgically treated animals were housed individually in cages. At days 1, 2, 3, 4, 5, 7, or 14 after wounding, the mice were sacrificed and the wound tissue samples with some surrounding skin were excised. After fixing overnight in 10% buffered formalin and dehydrating in ascending concentrations of ethanol, specimens were bisected horizontally across the center of the wound and embedded in paraffin. Microtome sections (5 μm) were mounted on glass slides which had been either silanated (as described by Maples, 1985) or pretreated with albumin for in situ hybridization or immunohistochemical analysis, respectively.

To extract RNA from granulation tissue, a standardized experimental wound model (as described by Niinikoski and co-workers, 1971) was used. Viscose cellulose sponge (Säteri OY, Valkeakoski, Finland) was cut into 40-mm-long cylindrical pieces with a 3-mm-diam tunnel through the center. Silicone rubber discs were stitched onto both ends of the sponge to create dead space. After decontamination by boiling, the cylinders were implanted subcutaneously under the back skin of adult male Sprague-Dawley rats anesthetized with ether. Rats were killed 7 d after implantation and the cylinders were dissected free from surrounding tissue and frozen in liquid nitrogen. Material was stored at −70°C until used for RNA extraction similar to that described above for cell cultures.

Immunohistochemistry

Both unlabeled peroxidase–antiperoxidase (PAP) and avidin–biotin–peroxidase complex (ABC) techniques, as described by Sternberger (1986) and Hsu et al. (1981), respectively, were used to detect the syndecan epitope in wound tissues. Stainings shown in this report were made using the PAP technique unless otherwise stipulated in figure legends. The ABC protocol elicited no background staining and was, therefore, used to study syndecan expression in granulation tissue cells.

For both types of staining experiments, slides were deparaffinized, rehydrated and incubated 30 min in 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Nonspecific binding was inhibited by further incubation of the slides 30 min in 2% normal swine (PAP; Dakopatts, Glostrup, Denmark) or goat (ABC; Vector Laboratories, Inc., Burlingame, CA) serum in Tris-buffered saline, pH 7.4 (TBS). As the primary antibody, we used a rat mAb (281-2) that specifically recognizes the core protein of mouse syndecan (Jalkanen et al., 1985). These stainings were controlled by the use of rat IgG (Sigma Chemical Co., St. Louis, MO), by the use of another IgG_{2a} mAb Mel-14 specific for lymphocyte homing receptor (Gallatin et al., 1983) or by the omission of the primary antibody. Primary antibodies were dissolved in TBS containing 1% BSA (as were all the other antibodies used) and incubated on the slides overnight at 4°C.

The PAP protocol was then carried on by sequential incubations for 30 min at room temperature with rabbit anti-rat IgG, swine anti-rabbit IgG and rabbit PAP complex (all delivered by Dakopatts); and the ABC staining, by similar treatments with biotinylated goat anti-rat IgG (Jackson's Immunoresearch Laboratories Inc., West Baltimore, PA) and avidin-biotin-peroxidase complex (Vector Laboratories, Inc.). Between each antibody incubation, the slides were washed three times with TBS. Immobilized peroxidase was visualized by incubation with 0.5 mg/ml of substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB; Polysciences Inc., Northampton, UK) in TBS containing 0.7 mg/ml imidazole and 0.01% hydrogen peroxide for 5 min. Finally, the sections were counterstained with hematoxylin, dehydrated and mounted (Gurr; BDH, Poole, UK).

In Situ Hybridization

The cRNA in situ hybridization for paraffin sections was performed according to the method of Wilkinson et al. (1989). A 535-bp SacI–KpnI fragment from the partial cDNA clone for mouse syndecan (PM-4) (Saunders et al., 1989) was subcloned (Vainio, S., M. Jalkanen, A. Vaahtokari, C, Sahlberg, M. Mali, M. Bernfield, and I. Thesleff, manuscript submitted for publication) into a riboprobe pGEM-4Z vector (Promega Biotec, Madison, WI). The cloned plasmid containing the insert was linearized with EcoRI or HindIII enzymes and antisense or sense transcripts were produced from complementary strands with T7 or SP6 polymerases in the presence of ^{35}S-UTP (Amersham International, Willshire, UK), respectively. The maximal length of the transcripts was reduced to <200 bp with alkaline hydrolysis; and those fractions with the highest specific activity were collected, precipitated and solubilized in hybridization buffer. Pretreated slides were hybridized overnight at 50°C, and the procedures to remove nonspecific binding of the probe (including high stringency washings and ribonuclease A treatment) and autoradiography were done, as previously described (Wilkinson et al., 1989).

Northern Blot

RNAs from cultured cells and granulation tissue were isolated by CsCl density centrifugation (Chirgwin et al., 1979). Purified RNA samples were fractionated on 1% formaldehyde-agarose gel, transferred to Gene Screen Plus membrane (New England Nuclear, Boston, MA) and hybridized with multiprime labeled (Amersham International) 1.0-kb HindII fragment of PM-4 (Saunders et al., 1989). Although this probe contains a sequence of

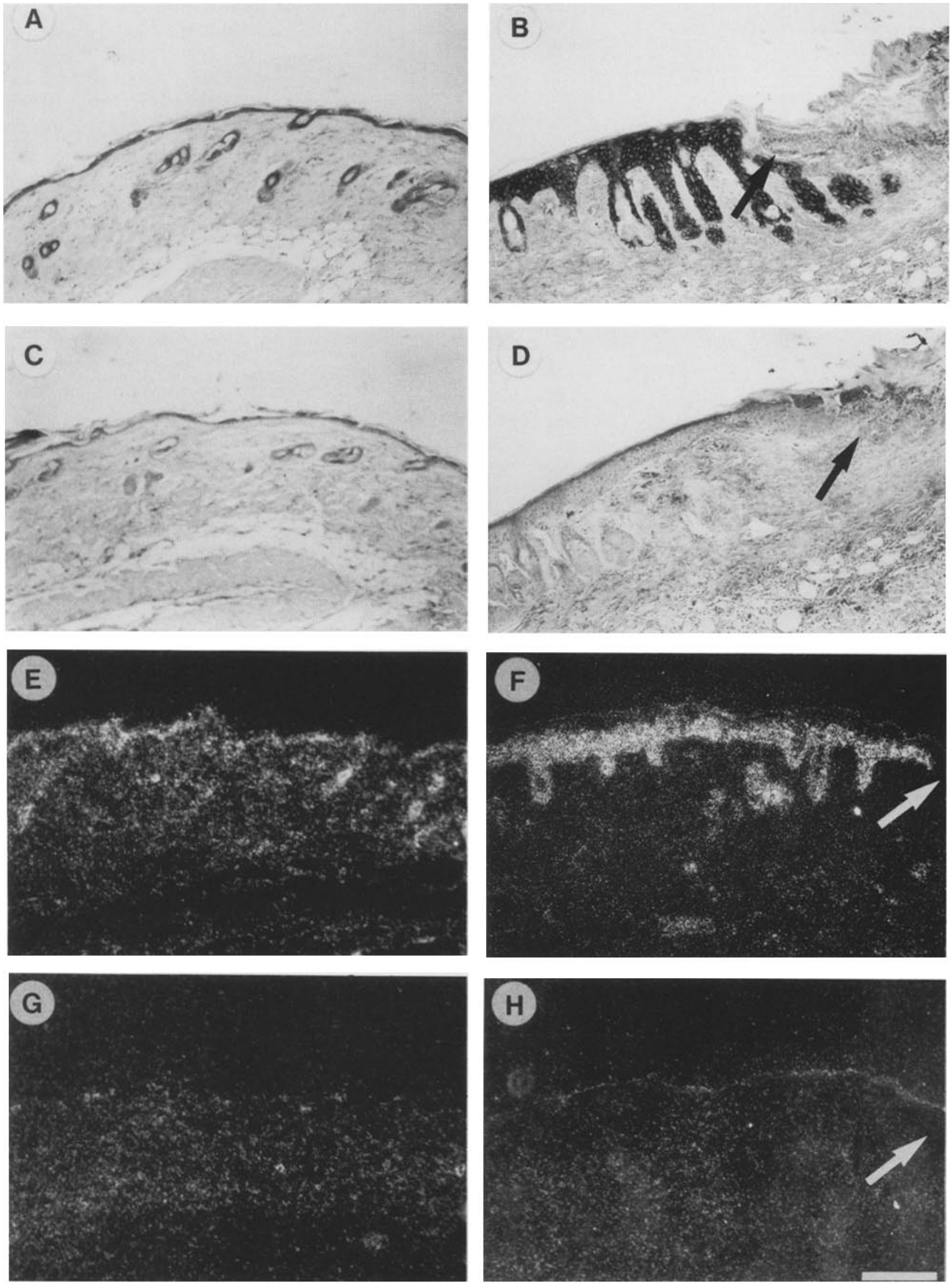

Figure 1. Syndecan expression in normal and wounded skin. Paraffin sections of normal mouse skin (*A*, *C*, *E*, and *G*) and 3-d-old sutured wound (*B*, *D*, *F*, and *H*) were immunostained with mAb 281-2 (*A* and *B*) or with nonspecific rat IgG (*C* and *D*), or were analyzed by in situ hybridization using antisense (*E* and *F*) or sense (*G* and *H*) constructs of syndecan-specific RNA-probes. Arrows point to the wound edge. Bar, 300 μm.

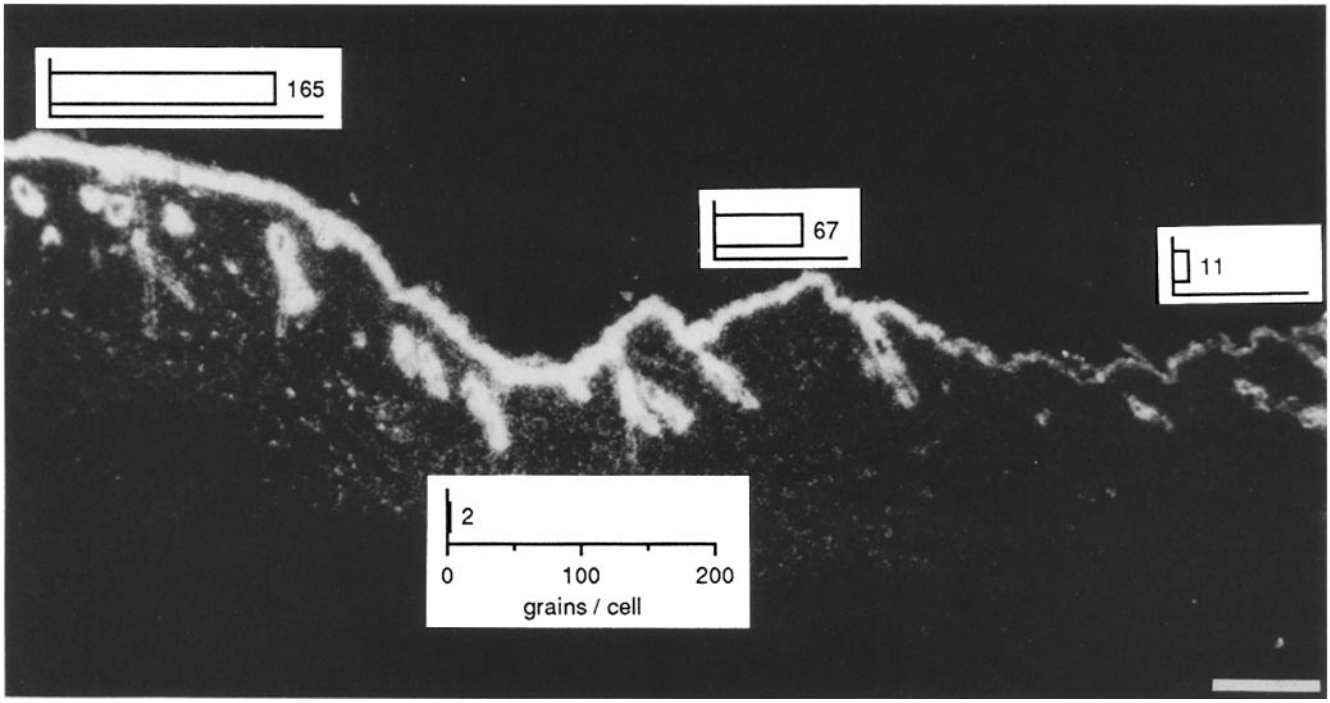

Figure 2. Quantitation of syndecan mRNA in tissues around healing wound. In situ hybridization grains per cell in peripheral epidermis (11 grains/cell) and dermis (2 grains/cell) as well as in moderately (67 grains/cell) and strongly (165 grains/cell) proliferating epidermis are shown. An open wound is located to the left. Bar, 300 μm.

mouse syndecan cDNA it was shown to detect also readily RNA from rat granulation tissue. This is so, because the HindII fragment of PM-4 includes regions coding for the cytoplasmic and transmembrane domains that have been discovered to be highly conserved between murine (Saunders et al., 1989), human (Mali et al., 1990) and hamster (Kiefer et al., 1990) syndecans. After hybridization, the filter was washed at 60 or 65°C in 2× SSC plus 1.0% SDS and autoradiographed, resulting in the detection of identical mRNA patterns. The use of the whole PM-4 as a probe resulted in the detection of the same mRNAs but gave reduced signal, as observed earlier between human and mouse syndecans and their cDNAs (Mali et al., 1990).

Results

Immunohistological and In Situ Localization of Syndecan in Normal Skin

In normal skin, mAb 281-2-positive staining has been found surrounding cells of epidermis and hair follicles (Hayashi et al., 1987; Jalkanen et al., 1988). Similar results were also obtained using the 281-2 antibody and either the PAP (Fig. 1 *A*) or the ABC (not shown) techniques. Immunohistochemical studies to detect syndecan may provide a somewhat limited picture of syndecan expression, because these techniques are based on the detection of few epitopes. In situ hybridization with syndecan specific cRNA probes, on the other hand, could reveal more information about syndecan expression in different issues. In this study, we confirmed that in normal skin, localization of syndecan transcripts is restricted to epidermis and to hair follicles (Figs. 1 *E* and 6 *C*), indicating that previous 281-2 stainings, at least in this tissue, had revealed correct expression sites for syndecan. This similarity between the immunohistochemical and in situ hybridization patterns also suggests that in cells in which the syndecan gene is transcribed, syndecan mRNA is further translated into protein. No specific signal was observed in the control sections that were probed with control antibodies (Fig. 1 *C*) or sense construct of the cRNA probe (Fig. 1 *G*).

Increased Syndecan Expression by Proliferating and Migrating Keratinocytes Adjacent to Wound

Our model for studying epidermal behavior within the wound consisted of a simple incision of the skin, followed by sample collection at 1 or 2 d intervals for immunohistological and in situ stainings. Analysis of these samples revealed increased intensity of syndecan staining around migrating and proliferating epidermis (Fig. 1 *B*), compared with the periphery of the same tissue section (Fig. 1 *A*). At the mRNA level, the induction of syndecan expression was even more evident. Fig. 1 *F* shows an in situ hybridization analysis of a section near the wound edge. It can be seen that cells of the epidermis and hair follicles demonstrated a strongly enhanced signal with syndecan anti-sense cRNA, when compared to those cells located more peripheral to the incision site (Fig. 1 *E*). This increase resulted from a higher syndecan mRNA copy number, as epidermal cells near the wound site contained severalfold greater grain number than those cells located more distal to the wound (Fig. 2). According to these observations, it is possible to conclude that the increased quantity of proteoglycan at the cell surface of keratinocytes near the wound edge was a consequence of accumulated syndecan transcripts. Negative controls for immunostaining (Fig. 1 *D*) and for in situ hybridization (Fig. 1 *H*) experiments of wounded skin gave no specific signal.

At higher magnification (Fig. 3) it can be seen that the proliferating epidermis, as a result of skin incision, was thoroughly 281-2 positive (Fig. 3 *A*); but syndecan mRNA production was restricted to the more basally-located cells (Fig. 3 *C*). Hayashi et al. (1987) have described that syndecan stain-

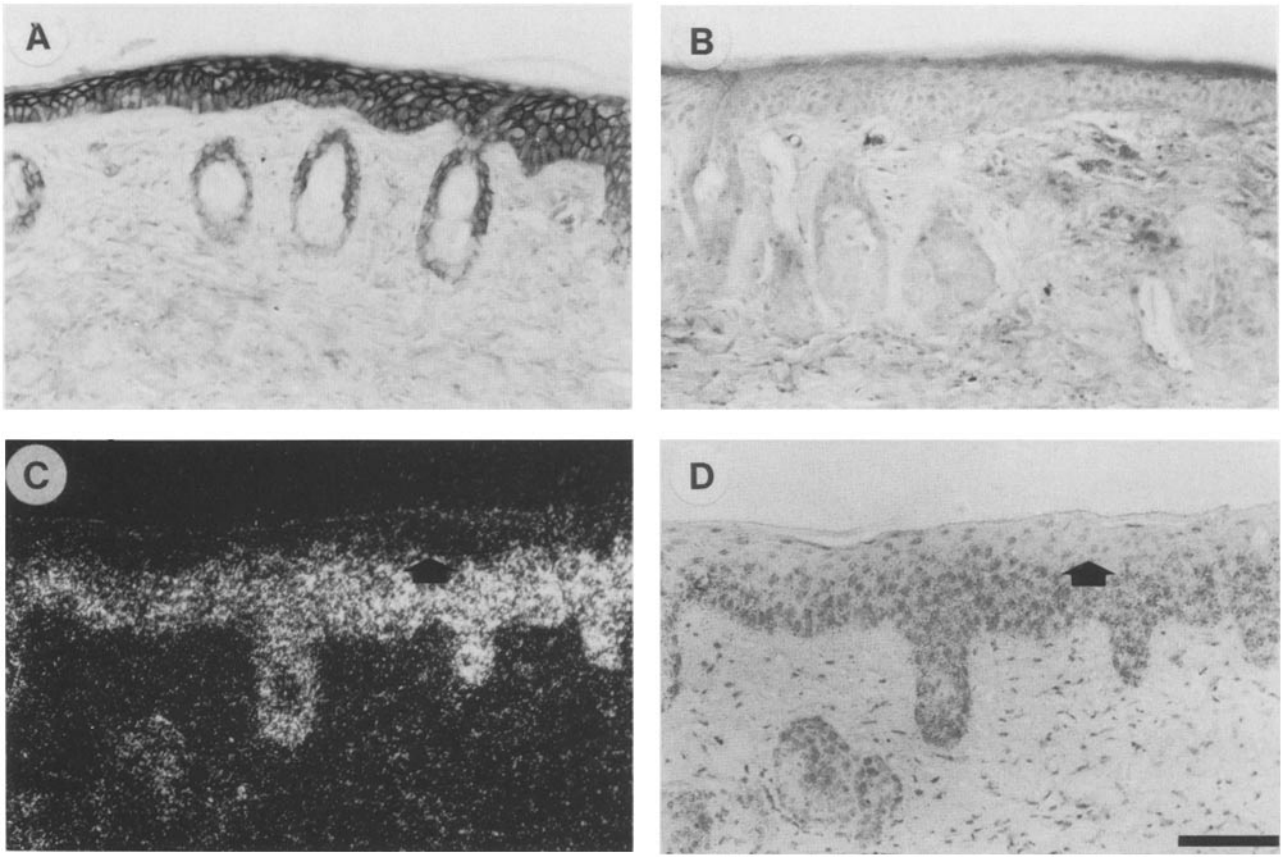

Figure 3. Localization of syndecan in the proliferating epidermis. Sections from 3-d-old sutured wounds were stained for immunohistochemistry (*A* and *B*) with mAb 281-2 (*A*) or with nonspecific rat IgG (*B*), and for in situ hybridization with syndecan anti-sense cRNA (*C*). Corresponding bright field is shown in D. Incision site is located 0.5–1.0 mm to the right (*A*, *C*, and *D*) or to the left (*B*) of the shown areas, resulting in a signal intensity gradient toward the wound. Arrows indicate the boundary of syndecan mRNA-negative and -positive cell layers of the epidermis. Bar, 150 μm.

ing in stratified squamous epithelia is most intense in basal cell layers, that expression is gradually decreased towards the surface and that few of the most superficial cells are totally negative. This gradient of syndecan expression was occasionally found also in our immunostainings of thick proliferating epidermis. However, the strict border between cells with positive or negative signal for syndecan mRNA, almost midway the epidermis (Fig. 3 *C*), was never seen with immunostainings. Therefore, syndecan in the uppermost cell layers must result from previous synthesis of syndecan by more basally located cells and their subsequent migration to a new more peripheral location. The wound site in Figs. 3, *A*, *C*, and *D*, located 0.5–1.0 mm right from the panels shown, and syndecan expression is seen to gradually increase towards the wound. Fig. 3 *B* is an immunohistochemical control staining and Fig. 3 *D* is a bright-field photograph of the same site shown with dark field optics in Fig. 3 *C*.

Expression of Syndecan at Different Stages of Wound Healing

In general, the induction of syndecan expression, both at the protein (not shown) and at the mRNA (see Fig. 5 *A*) level was clearly evident already a day after wounding and still observable, although considerably diminished, at our last time point of two weeks (not shown). Maximal induction was seen at the time of strongest keratinocyte proliferation and migration 3 to 5 d after wounding, depending on whether the wound was primarily sutured (Fig. 1) or not (see Fig. 7 *A*).

The leading cells in the epidermal sheet migrating below the scab stained positively with mAb 281-2, but the strongest signal was found at sites of rapid cell division adjacent to the wound margin (Fig. 4 *A*). At the time of epithelial merging, the cells at the contact site seemed to be poor syndecan expressors (Fig. 4 *B*); but the epidermis at the point of original incision after recovery and merging revealed very intense syndecan expression (Fig. 4 *C*).

The in situ hybridization experiments (Fig. 5) remarkably duplicated the discoveries made by immunostaining. The leading cells of the migrating epithelium showed active transcription of syndecan gene (Fig. 5 *A*); but again, the cells behind the leading edge revealed an even higher expression of syndecan mRNA. At the time of the first epithelial recontact, those cells most probably derived from the basal cell layers of the epidermis or from cells in hair follicles (Krawczyk, 1971; Stenn and Depalma, 1988) were negative for syndecan gene transcription (Fig. 5, *C* and *D*). However, similar to the immunostainings, the epidermal cells at the time of epithelial proliferation after merging displayed very intense expression of syndecan (Fig. 5, *E* and *F*). The most extensive syndecan mRNA production levels in these more advanced wounds was found at the site of incision and gradually diminished towards the more normalized periphery (Fig. 5, *E* and *F*).

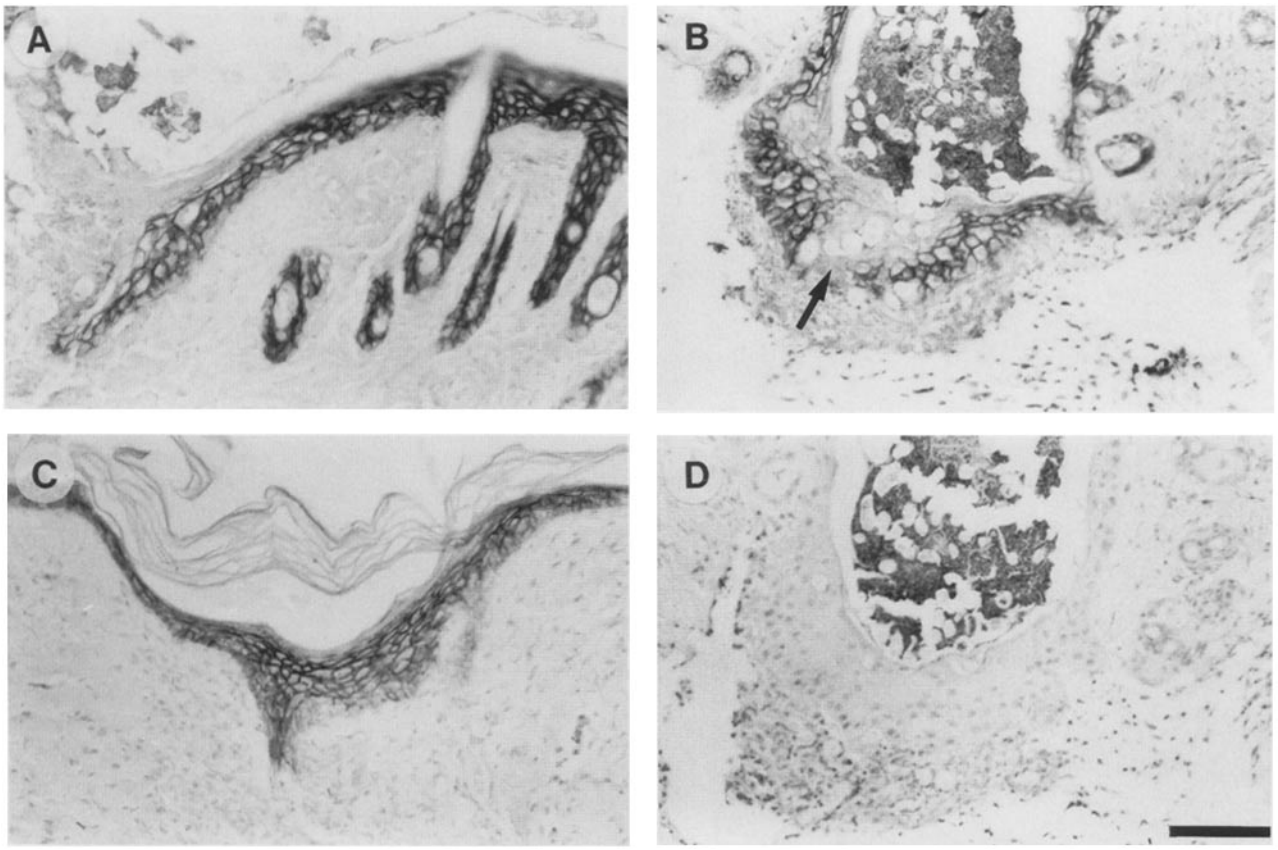

Figure 4. Immunohistochemical localization of syndecan at different stages of wound healing. All wounds were primarily sutured. (*A*) A wound in which the epidermal sheet is seen migrating under the scab. (*B*) A wound section showing merging epithelial sheets. Arrow indicates cells, which are already in contact and have lost their syndecan expression. (*C*) 5-d-old wound with heavy expression of syndecan at the original incision site. *D* shows control staining (rat IgG) for a section adjacent to that shown in *B*. Bar, 150 μm.

Syndecan Induction Correlates to Increased Epithelial Proliferation

Syndecan production in keratinocytes was strongly enhanced after wounding of the skin. Whether this stimulation was a consequence of, or a prerequisite for, cell migration or proliferation is difficult to judge. As shown earlier in Figs. 4 and 5, the epithelial thickening of the epidermis correlated well with increased syndecan expression. Moreover, the hair follicle cells adjacent to the wound edge were also induced to synthesize manyfold quantities of syndecan (Fig. 6 *B*) and its mRNA (Fig. 6 *D*) when compared with the more peripheral counterparts (Fig. 6, *A* and *C*). These cells reacted to the wounding process by enhanced proliferation, rather than migration. On the other hand, the first migrating epidermal cells, which have been suggested not to divide (Stenn and Depalma, 1988), clearly expressed less syndecan (Figs. 4 *A* and 5 *A*). These findings imply, that syndecan induction correlates primarily to increased epithelial proliferation rather than to epithelial migration, suggesting an important role for syndecan in the morphological differentiation process of epidermis based on proliferation.

Syndecan Expression by Vascular Endothelium of Granulation Tissue

An unsutured wound healed by secondary intention and was characterized by formation of large amounts of granulation tissue that fills the open space between the wound edges (Fig. 7). When the margins were carefully attached together (e.g., by suturation) the generation of granulation tissue was considerably less prominent (Figs. 1 and 4). In a 4-d-old wound which was left uncovered, the epithelial sheet migrated beneath the scab and, as shown by in situ hybridization, strongly expressed syndecan mRNA (Fig. 7, *A* and *B*). At the same time, some scattered positive signals were also observed in the newly formed granulation tissue beneath and in front of the migrating keratinocytes (Fig. 7, *A* and *B*). With higher magnification, the grains colocalized within single cells (Fig. 7 *C*). No corresponding signal below the epidermal sheet was found with in situ hybridization in wounds of the same age that had been primarily sutured (Fig. 1 *F*) or in unsutured wounds before day 2 (Fig. 5 *A*) or after day 5 (not shown) postoperatively.

Unfortunately the lack of clarity in the morphological details of the sections treated for in situ hybridization analysis disallowed identification of the type of the positive cells involved. In order to more accurately approach this problem, we selected similar sections, as shown in Figs. 7, *A* and *B*, for ABC peroxidase staining with mAb 281-2. By this method, syndecan expression was detected in 4-d-old granulation tissue around the endothelial cells of growing capillaries (Fig. 7 *D*) and was evident at the basal site of these cells. Surprisingly, positive staining was selective; and some capillaries were negative in the same section (Fig. 7 *D*). Positive staining was observed neither in endothelial cells of peripheral dermis (not shown), of normal skin (Fig. 1 *A*) nor in

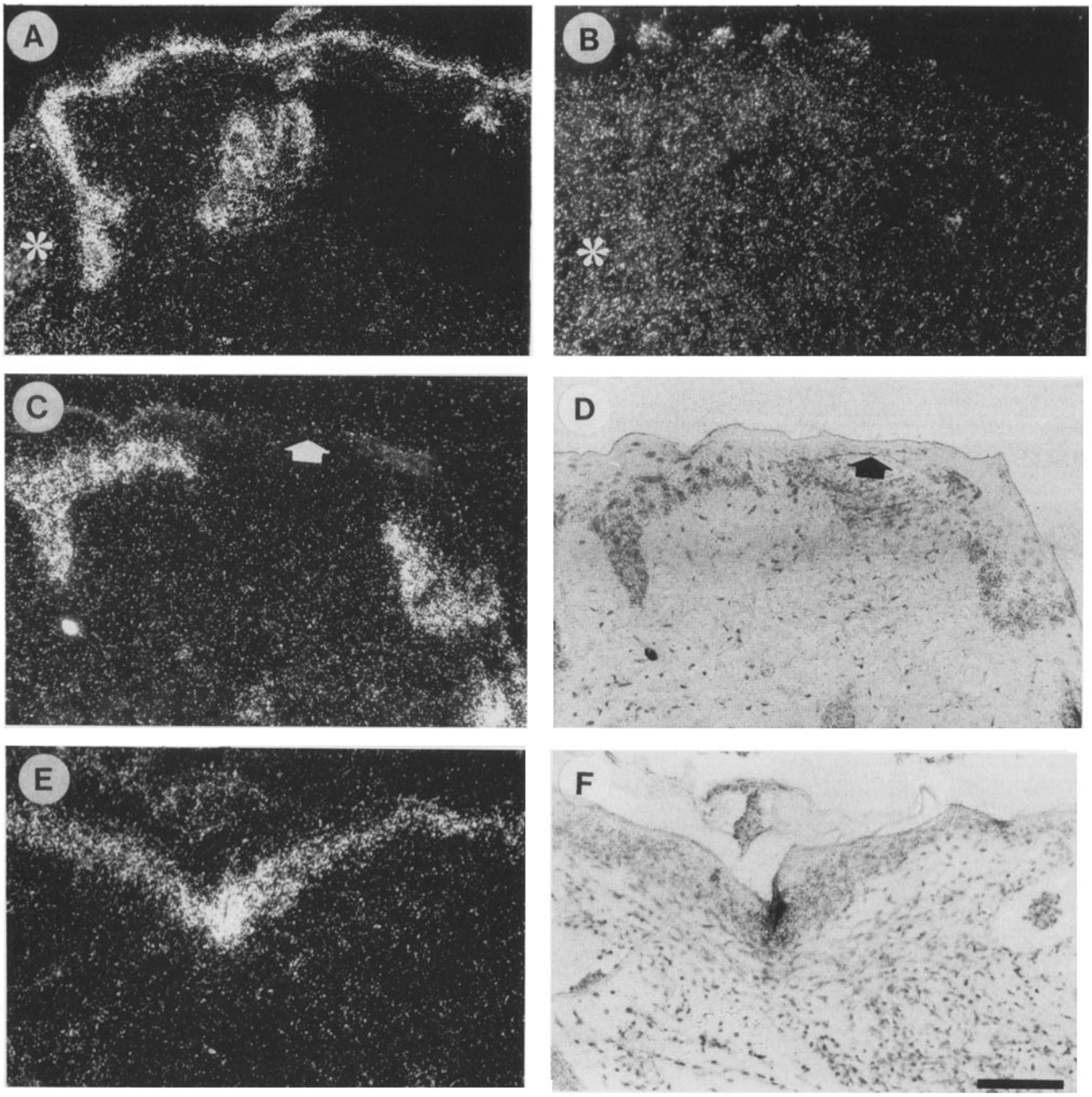

Figure 5. Localization of syndecan gene activity at different stages of wound healing. (*A*) Epidermal sheet is protruding under the scab (*asterisk*) in 1-d-old open wound. (*B*) Control section for A probed with a sense construct. (*C*) First merging epidermal cells (arrow) derived from opposite margins of 3-d-old sutured wound. (*D*) Bright field photograph of the same field as shown in C. (*E*) Proliferating epidermis at the original incision site in a 5-d-old sutured wound. The level of mRNA production is gradually decreasing towards the already normalized epidermal region. (*F*) Bright-field photograph of the same field as seen in E. Bar, 150 μm.

dermis of the sutured wounds (Figs. 1 *B* and 4). Finally, we selected a few good sections containing typical growing capillaries and hybridized serial sections with anti-sense and sense syndecan cRNA probes. As seen in Fig. 7, *E* and *F*, these stainings clearly revealed syndecan mRNA-positive structures resembling vascular endothelia. We cannot, however, totally exclude the possibility that those cells in the granulation tissue expressing syndecan mRNA would consist only of endothelial cells. As further conjuncture, some other cell types synthesizing an immunologically different syndecan molecule could be partly responsible for the findings at the mRNA level: For example 3T3 fibroblasts express syndecan in cell culture conditions (Fig. 8, lane *B*); and, moreover, differentiating mesenchymes, during organogenesis (Vainio et al., 1989*a*,*b*; Solursh et al., 1990).

To confirm that the granulation tissue was, in fact, really expressing syndecan we prepared Northern blots for material extracted from cylinders implanted subcutaneously into rats (see Materials and Methods). These cylinders, made from cellulose sponge, have been described to fill with tissue very much resembling the granulation tissue generated during normal wound healing (Viljanto and Kivikoski, 1962). This tis-

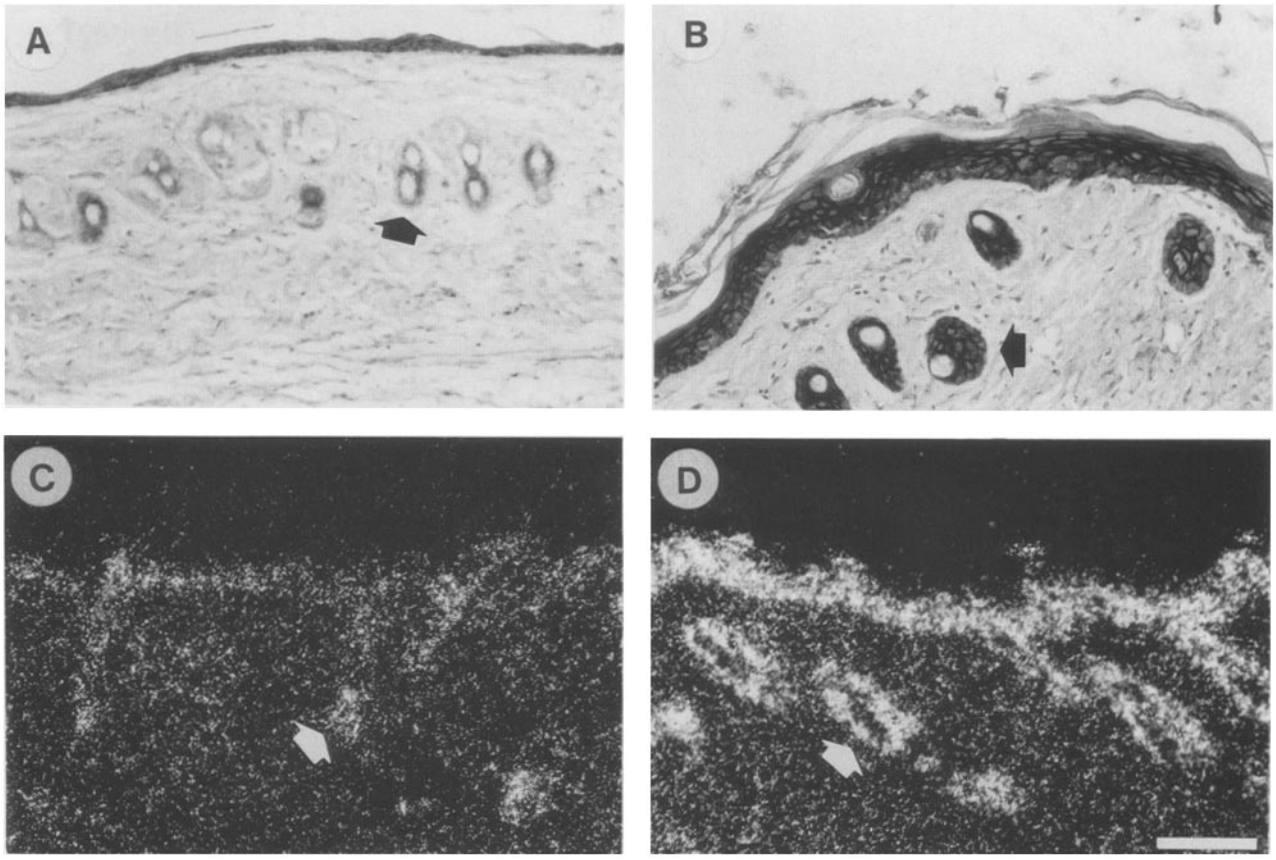

Figure 6. Expression of syndecan in hair follicles of normal skin and near the wound edge. *A* and *B* are immunostainings with mAb 281-2; and *C* and *D* are in situ hybridization, with syndecan antisense cRNA. (*A* and *C*) Normal peripheral skin. (*B* and *D*) Tissue near the wound edge. Arrows indicate the hair follicles. Bar, 150 μm.

sue produced the same species (2.6 and 3.4 kb) of syndecan mRNA (Fig. 8, lane *A*) in similar proportions as 3T3 mouse fibroblasts (Fig. 8, lane *B*) and as the original source of syndecan (Saunders et al., 1989), the NMuMG mouse epithelial cells (Fig. 8, lane *C*). These observations indicate that the signal in granulation tissue was produced by the same type of mRNA and not by its variants.

Discussion

The importance of cell–matrix interactions and cell–cell contacts for organ and tissue formation has been recognized for several decades. Syndecan, a cell surface proteoglycan (Saunders et al., 1989; Mali et al., 1990) is one of the numerous cell surface molecules involved in these interactions. Its biochemical properties (Rapraeger et al., 1985; Jalkanen et al., 1987; Saunders et al., 1989) and its expression during organ formation, where it follows morphogenetic rather than histological boundaries (Thesleff et al., 1988), support a hypothesis of important participation of syndecan in these interactions, both as a matrix anchor and as a cell adhesion molecule. As tissue regeneration resembles normal development, it is reasonable to assume that mechanisms regulating normal development could also play an important role during, for example, reconstruction of wounded tissues. We have shown in this paper that the increased proliferation and migration of epidermal cells of skin after incisional wounding coincides with the enhanced expression of syndecan, suggesting that syndecan is needed for the regeneration of wounded skin. Expression patterns of syndecan during wound healing suggest that syndecan could be very important in epithelial adhesion. Syndecan is also known to bind growth factors, like bFGF (Kiefer et al., 1990), and it may, therefore, also participate in the modulation of growth factor response at the site of tissue injury.

Syndecan Expression and Epithelial Behavior during Wound Healing

Recovery of the protective epithelial layer of the skin after incisional wounding requires both proliferation and migration of the keratinocytes near the wound margin. The precise epidermal sites at which these two processes take place are morphologically distinguishable from each other (Clark, 1989). The most active cell division, on the other hand, has been supposed to localize slightly peripheral to the wound margin where, on the other hand, the most rapidly migrating keratinocytes are found (Krawczyk, 1971). Also supporting the idea of independent progression of keratinocyte proliferation and migration is the fact that the motility of the epithelial sheet is not affected under conditions that inhibit cell division (Dunlap and Donaldson, 1978; Gipson et al., 1982). Epithelial cells of the epidermis, which had a high tendency to migrate and were first cells to merge at the incision site, were found to be syndecan negative. Also the distal cells of the migrating epithelial sheet showed less expression than

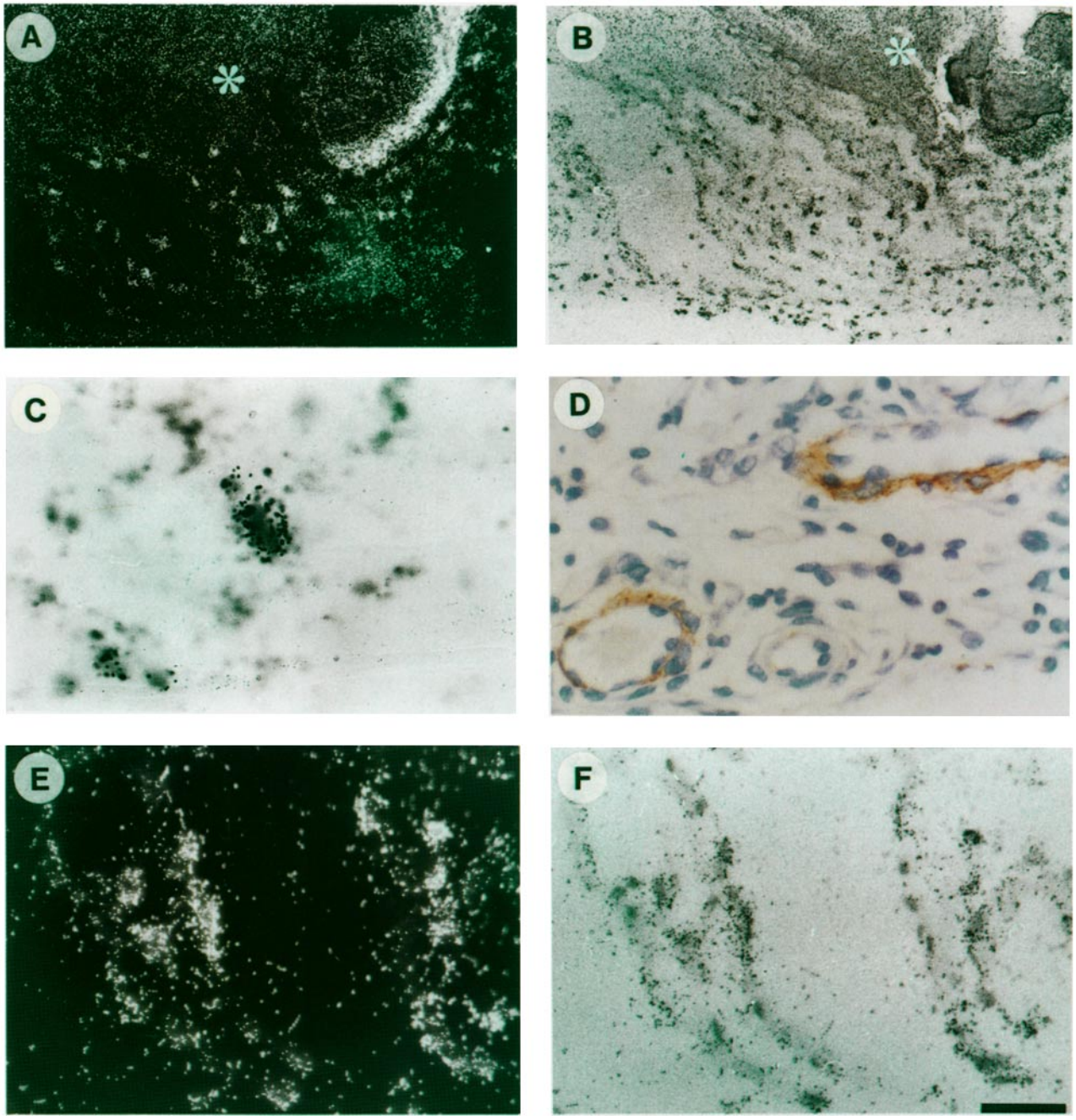

Figure 7. Induction of syndecan expression in the granulation tissue. All stainings are from 4-d-old open wounds. (*A*) In situ hybridization experiment, showing a syndecan-specific signal in the migrating epidermal sheet and also in some scattered points beneath and in front of the sheet. (*B*) Bright field photograph of the same section as shown in A (*asterisks* indicate the location of scar). (*C*) In situ hybridization grains in the granulation tissue colocalize with single cells. (*D*) Using ABC peroxidase staining, the syndecan (*brown color*) in granulation tissue is localized to endothelial cells of some growing capillaries. (*E*) In situ grains are found in cells forming vessels. (*F*) Bright field of E. Bars: (*A* and *B*) 300 μm, (*C*) 30 μm, (*D*, *E*, and *F*) 50 μm.

proliferating nonmigratory cells. This could indicate that migrating cells loose their cell surface syndecan. It has been previously shown that the ectodomain of syndecan can be shed from epithelial surfaces by cleavage at a trypsin-sensitive site adjacent to plasma membrane and, further, that rounding of epithelial cells can stimulate syndecan shedding (Jalkanen et al., 1987). Indeed, these two phenomena could be linked together, and could mean that migrating epithelial cells may actively lose syndecan-mediated adhesion, to regain it later during the stratification and differentiation accompanying with cell proliferation. The increased proteolytic activity, which has been thought to aid the protrusion of the epidermal sheet under nonviable tissue (Grøndahl-Hansen et al., 1988), may also contribute to enhanced shedding of the ectodomain.

Syndecan production, however, was clearly induced in the nonmigratory cells of thickening epidermis and hair follicles. Regulation of syndecan expression has been shown to

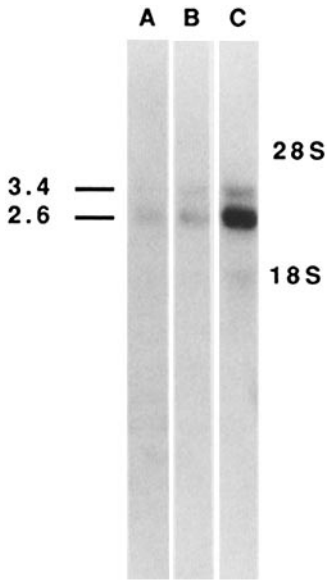

Figure 8. Northern blot analysis of granulation tissue RNA with a syndecan-specific probe. Total RNAs (10 μg) from viscose cellulose sponges containing granulation tissue (lane *A*; see Materials and Methods), from cultured 3T3 cells (lane *B*) and from NMuMG cells (lane *C*) were size-separated, transferred to a GeneScreen filter, probed with a HindII fragment of PM-4 and washed in 2× SSC plus 1% SDS at 60°C. Syndecan-specific mRNAs are indicated (2.6 and 3.4 kb), as well as both ribosomal RNAs (18 and 28S).

correlate to the regulation of epithelial cell morphology both in vitro (Leppä et al., 1991) and in vivo (Hayashi et al., 1988). The modified extracellular environment, that might stimulate the synthesis of appropriate matrix receptors, may be involved. The provisional matrix under the advancing epidermal sheet has been described to consist of, for example, fibronectin (Clark, 1988; ffrench-Constant et al., 1989), type V collagen (Stenn et al., 1979) and tenascin (Mackie et al., 1988), ECM molecules that have also been demonstrated to be ligands for syndecan (Elenius et al., 1990). Interestingly, the basement membrane components, laminin and type IV collagen, which do not bind syndecan (Elenius et al., 1990), have been shown to devoid the matrix below the leading edge of epithelial cells (Stanley et al., 1981; Fujikawa et al., 1984). These findings suggest that ECM molecules assembling under the migratory epithelium could stimulate expression of syndecan because of its strong tendency to interact with stromal components.

The mediator that induces the expression of syndecan in wounded epidermis may also be one of the many growth factors released in the area. This was supported by the finding that induced syndecan expression correlated better to keratinocyte proliferation than to migration, thus being a consequence of soluble factors rather than changed ECM. In our preliminary in vitro studies, we found that bFGF and TGFβ (transforming growth factor beta) have a stimulatory effect on syndecan gene expression when applied simultaneously to cell culture medium (Elenius, K., A. Määttä, and M. Jalkanen, unpublished results). These two growth factors are thought to take part in the regulation of tissue regeneration during wound healing (Mustoe et al., 1987; Burgess and Maciag, 1989). Furthermore TGFβ is also known to alter the expression of other matrix receptors, such as the integrins (Heino et al., 1989).

Some developing capillaries of the granulation tissue of open wounds were surprisingly also observed to transiently express syndecan mRNA and mAb 281-2 epitope. Because syndecan has been shown to bind bFGF (Kiefer et al., 1990) and, moreover, bFGF has been described to actively stimulate angiogenesis (Folkman and Klagsbrun, 1987), it is very tempting to speculate that the formation of new capillaries in the granulation tissue could, at least in part, be generated by the increased syndecan-modulated bFGF response. In vitro studies have shown that wounded endothelial cells increase their proteoglycan expression (Kinsella and Wight, 1986), but no information exists for the involvement of syndecan in these changes.

Role of Syndecan in Tissue Regeneration

Syndecan has been proposed to serve in three different functions: (*a*) as a matrix receptor for ECM (Koda et al., 1985; Saunders and Bernfield, 1988); (*b*) as a cell–cell adhesion molecule (Hayashi et al., 1987; Jalkanen et al., 1988); and (*c*) as a growth factor binding molecule (Kiefer et al., 1990). Each of these putative roles of syndecan, in and of themselves, are important for the growth and integration of tissues. It is clear that in healing skin wounds, however, syndecan functions co-operatively with several other adhesion molecules. The expression of the fibronectin receptor, a member of the integrin family, has been shown to be regulated during wound healing (Clark, 1990). Many other integrins (Larjava et al., 1990; De Luca et al., 1990) as well as cadherins (Takeichi, 1990), are known to function as adhesion molecules for epidermal cells. Regulation of cell adhesion together with growth factor responses may represent one of the principal mechanisms whereby to regulate coordinated growth and differentiation of cells during normal development and regeneration. Our recent findings with proliferating malignant cells of skin tumors indicate that such cells can proliferate but fail to maintain differentiated phenotype when syndecan expression is suppressed (Inki, P., F. Stenbäck, L. Talve, and M. Jalkanen, manuscript submitted for publication), suggesting a key role for syndecan in the normal growth and regeneration processes.

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