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BASAL MEMBRANE HEPARAN SULPHATE PROTEOGLYCAN EXPRESSION DURING WOUND HEALING IN HUMAN SKIN

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SUMMARY

Heparan sulphate proteoglycans (HSPGs) are integral components of the basement membrane (BM) in various tissues. HSPGs are important in the assembly and structure of the BM, and their putative functions include regulation of basement membrane permeability, binding of growth factors, and a role in cellular adhesion. In this study the expression of HSPG was examined during wound healing in human skin, using monoclonal antibodies (MAbs) that recognize the HSPG core protein and two different heparan sulphate (HS) epitopes, and the dynamics of HSPG expression were investigated in relation to epidermal cellular proliferation and permeability of the BM. Healing of excisional wounds in healthy volunteers was studied from day 0 up to 1 year. Intact human skin showed strong continuous staining of the dermo-epidermal BM and the vascular BM with all MAbs. Up to day 4 after wounding, staining for HSPG was absent under the ingrowing epidermis, with any of the MAbs, indicating that no complete BM was present. From day 7 onwards, the BM of the neo-epidermis showed positive staining for the HSPG core protein and a low sulphated HS epitope, and after day 14, the staining intensity was similar to normal skin. The staining patterns of these HSPG epitopes were similar to that of laminin. The staining pattern with a MAb against an epitope in the highly sulphated part of HS was found to be distinct from the other BM markers studied. This epitope was absent under the neo-epidermis up to 2 months after wounding. One year after wounding, the epitope was found to be present again. We observed that only in the time period between 2 months and 1 year had the epidermis normalized with respect to the number of cycling cells and the absence of high molecular weight plasma proteins. These findings suggest a correlation between normalization of epidermal proliferation, BM permeability, and regeneration of BM HS. It is proposed that complete BM maturation following skin wounding is a slow process and may account for the epidermal abnormalities that persist for a considerable period of time after wound healing.

KEY WORDS—heparan sulphate; proteoglycan; wound healing; epidermal proliferation; basal membrane; human skin; immunohistochemistry

INTRODUCTION

Proteoglycans (PGs) are macromolecules with a ubiquitous tissue distribution. They each contain a core protein with one or more covalently bound glycosaminoglycan (GAG) side-chains. The amino acid sequences of many PG core proteins have been obtained by cloning of their corresponding cDNAs. Some of these PGs, such as perlecan, syndecan-1, and epican (a member of the CD44 family), contain heparan sulphate (HS) side-chains. These heparan sulphate proteoglycans (HSPGs) are found both on cell surfaces and in basal membranes (BMs), where they function as a structural element and are involved in regulatory functions. BM-associated PGs contribute to the charge-dependent permeability of BMs, especially in the glomerular BM. Several studies indicate that BM HSPGs are capable of binding a wide variety of biologically active proteins including growth factors such as basic growth factor (bFGF). Interaction of BM HSPGs with other BM components such as fibronectin, laminin, and collagen type IV accounts for their role in the assembly and integrity of the BM.

Increasing amounts of data have become available on the localization and function of HSPGs in stratified epithelia. Most studies, however, describe only the presence of cell-surface HSPGs. Syndecan-1 is, for example, localized over the entire surface of keratinocytes, especially in the suprabasal layers, whereas the basal layer shows modest expression. Antibodies that recognize the core proteins of CD44 and biglycan bind to the intercellular space of the entire epidermis.

At present, few data are available on the localization and function of BM HSPGs in human skin. We have previously investigated the presence of BM HSPGs in normal human skin and in epidermal tumours, showing that HSPGs are associated with the basal membranes of the epithelia and dermal capillaries. Murdoch et al. also found strong immunoreactivity for perlecan at the dermal/epidermal junction of normal human skin. A faint reaction was observed in fibrous strands leading into the underlying dermis, the upper dermis, and the...
perivascular region. The keratinocytes were consistently negative. Recently we described the immunoreactivity of HSPGs during the first 2 weeks of wound healing in normal human skin. Using an antibody directed against the HS side-chain and comparing it with the BM component laminin, we observed a disparity in the time of reappearance during restoration of the BM. Considering the variety of functions attributed to BM HSPGs, it is likely that the expression levels and the quality of HSPGs could affect several aspects of wound healing, such as re-epithelialization, epidermal growth control, proliferation and BM permeability, and the putative functional implications are discussed.

**MATERIALS AND METHODS**

**Antibodies**

MAbs directed against the HSPG core protein (JM-72) and the heparan sulphate side-chain (JM-403 and JM-13) were developed as previously described (Table I), and directed against the core protein of HSPG from human glomerular BM. Analysis of the immunofluorescence pattern of MAb JM-72 suggests glomerular BM HSPG to be related to agrin, rather than to perlecan. Anti-HS MAb JM-13 reacts with an HS epitope containing O-sulphates. This is based on the unpublished observation that MAb JM-13 does not bind to the non-sulphated *Escherichia coli* K5 capsular polysaccharide, which has the same basic structure as the unmodified biosynthetic precursor of heparin/HS, but strongly binds to chemically O-sulphated K5. This latter K5 derivative contains an average of 1-2 O-sulphate (but not N-sulphate) groups per disaccharide unit. The location of the O-sulphates would presumably primarily involve C-6 of the glucosamine unit, along with C-2 and/or C-3 of the glucuronic acid units. The epitope fine specificity of anti-HS MAb JM-403 has been published and involves an N-unsubstituted glucosamine unit along with an N-sulphated glucosamine unit. Iduronate residues and O-sulphates interfere strongly with antibody binding. The epitope is exclusively found in low sulphated (<1 sulphate per disaccharide unit) HS isoforms.

A MAb directed against laminin and a polyclonal Ab directed against human fibrin/fibrinogen were obtained from DAKO Corporation (Carpinteria, U.S.A.). A MAb to human Ki-67 antigen (MIB-1 clone) was obtained from Immunotech S.A. (Marseille, France). Rabbit anti-mouse immunoglobulins conjugated with horseradish peroxidase (RAMPO) and swine antirabbit immunoglobulins conjugated with horseradish peroxidase (SWARPO) were obtained from DAKO Corporation (Carpinteria, U.S.A.).

**Tissues**

To study wound healing in normal human skin, the following model was applied to healthy volunteers (eight subjects aged 25–55 years, mean 42 years). At day 0, in each subject, four wounds were made under local anaesthesia on the outside of the upper arm using a biopsy punch of 3 mm diameter. The wound depth was approximately 1 mm. At days 1, 2, 4, 7, 14, 60, and 365 after wounding, punch biopsies of 4 mm diameter were taken comprising the previously made wounds and some surrounding tissue. The wound depth was approximately 5 mm. Three to four biopsies were studied per time point. Permission from the local medical-ethical committee was obtained prior to the experiment.

**Immunohistology**

Each biopsy was snap-frozen in liquid nitrogen and stored at −80°C until further use. Cryosections were prepared using standard procedures. Sections were incubated with MAbs JM-72, JM-403, JM-13, MIB-1, and anti-laminin as described previously. After incubation with RAMPO or SWARPO, the sections were developed with aminoethyl carbazole as the chromogenic substrate. All sections were counterstained with haematoxylin.

**Scoring of the sections**

BM staining was classified as continuous (+) or patchy (±) (see Table II). No attempt was made to quantify staining intensity. Ki-67 staining was quantified by counting the number of moderately and strongly positive basal and suprabasal nuclei per unit of 100 basal cells.

**RESULTS**

**Histopathology of the wounds**

Routine haematoxylin and eosin (H & E) staining of the biopsies showed the well-known features of

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unimpaired wound healing. A dense infiltrate of inflammatory cells was present shortly after injury, and the wound bed was filled with fibrinous material. Within 2 days after wounding, re-epithelialization had started and a sheet of ingrowing keratinocytes was visible. Up to 4 days after injury, polymorphonuclear leukocytes were prominently present. After 1 week, re-epithelialization was complete in nearly all wounds and only a few scattered polymorphonuclear leukocytes remained in the original wound bed. After 2 weeks, the keratinocytes had resumed their normal stratification. However, the epidermis still remained acanthotic and rete ridges were lacking. In the dermis, granulation tissue with mononuclear cells, fibroblasts, and endothelial cells was present. Up to 1 year after wounding the wound area could still be distinguished from the surrounding tissue by the absence of epidermal rete ridges, dermal fibroplasia, and the absence of elastic fibres.

**Immunolocalization of BM components during wound healing**

Figures 1 and 2 show the time course of expression of BM components after partial thickness wounding. Intact human skin gives identical continuous, linear staining of the dermo-epidermal BM and the BM of dermal vasculature for three MAbs against different HSPG epitopes (JM-403: low-sulphated HS; JM-13: high-sulphated HS; JM-72: core protein) and for an anti-laminin MAb (see Figs 1A and 2A: staining patterns with JM-403 and laminin MAbs are not shown). After wounding of the skin, no marked changes were noted in the staining patterns for any of the antibodies in the BM of the normal skin at the wound margins during the time course studied. During the re-epithelialization process (which starts within 2 days), the BM zone of the neo-epidermis was negative for all HSPG antibodies and the laminin antibody up to day 4 (Figs 1B and 2B). One week after wounding, when re-epithelialization is complete, weak linear staining with the JM-72, JM-403, and anti-laminin antibodies was seen in the BM zone of the neo-epidermis (not shown). Staining for JM-13 remained negative (not shown). From day 7 up to 1 year after wounding, staining for JM-72, JM-403, and anti-laminin was increased to normal intensities (Figs 2C and 2D). Remarkably, the JM-13 antibody, which recognizes a high-sulphated epitope of the HS side-chain, remained undetectable up to 2 months after wounding, indicating that in spite of the intact epidermis and nearly normal morphology, the BM is not completely restored (Fig. 1C shows absent staining after 14 days). Biopsies taken after 1 year revealed a staining pattern of JM-13 which was similar to that of normal skin (Fig. 1D). Table II summarizes the immunohistochemical data.

**Epidermal hyperproliferation and BM permeability during wound healing**

The structural integrity of the BM, and the presence of HSPGs in particular, could affect several properties of the neo-epidermis, taking into account the putative functions of HSPGs. To investigate whether the absence of high-sulphated HS epitopes correlated with growth alterations of the epidermis, we performed an immunohistochemical staining for the Ki-67 antigen, which is expressed by cycling cells. In a previous study we quantified the number of Ki-67-positive cells during normal wound healing from day 0 to day 14. Here we extended our observations up to 1 year. We found that the epidermis remained hyperproliferative up to 2 months after injury. In normal skin we found 14 ± 2 Ki-67-positive cells per unit BM, which were present both in the basal layer and in the first suprabasal layer (Fig. 3A). These cells probably represent the stem cells and the transit amplifying cell population. As shown before, the cycling population was dramatically increased in the neo-epidermis after wound closure at days 7 and 14. Here we found in addition that after 2 months the epidermis was still hyperproliferative (66 ± 45 positive cells per unit BM, see Fig. 3B). After 1 year, the number of Ki-67-positive cells in the epidermis overlying the former wound bed was normalized (16 ± 7 positive cells per unit BM, see Fig. 3C).

Previous studies have suggested that the negative charge of the basal membrane which is provided by the HS molecules is responsible for the selectivity and permeability of the glomerular BM. The BM of normal intact skin is thought to be relatively impermeable to high molecular weight plasma proteins. Here we used an anti-fibrin antiserum to detect fibrinogen in the epidermis, as a measure for changes in BM permeability during wound healing. Figure 4A shows that normal epidermis is virtually negative for fibrinogen, whereas the connective tissue shows positive staining. Obviously, in the early stages of wound healing, the wound bed is strongly positive for fibrinogen/fibrin (not shown). At day 14 after wound closure, we observed strong pericellular staining for fibrinogen in the epidermis (Fig. 4B). After 2 months, strong epidermal staining for fibrinogen was still observed in two biopsies.
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Fig. 1—Immunohistochemical staining with JM-13 MAb directed against a highly sulphated epitope in HS chains in normal human skin and after wounding (A) Normal skin shows a continuous, linear staining pattern along the entire dermo-epidermal junction (e—epidermis). (B) At day 4 after wounding the ingrowing neo-epidermis (ne) beneath the scab (s) is negative for the JM-13 antibody (arrows), whereas the dermo-epidermal junction at the wound edge is positive (arrowheads). The positive staining signal of the scab is caused by endogenous peroxidase of the inflammatory cells. (C) At day 14 after wounding, the neo-epidermis (ne) is still negative. However, 1 year after wounding (D), the epidermal BM is positive for JM-13 (e—epidermis). Note that the apparent positive staining in the basal layer of the normal epidermis is caused by melanin.

and weak staining in one biopsy. The staining varied within the epidermal compartment of the individual biopsies. After 1 year, staining for fibrinogen in the epidermis was faint to absent, as in normal skin (not shown).

DISCUSSION

In this study we investigated the expression of HSPGs in the dermo-epidermal BM during healing of partial thickness wounds in human skin. It is shown that basal membrane regeneration with respect to the expression of laminin and HSPGs is completed within 2 weeks after injury.

Although laminin and HSPGs are detected within 2 weeks after wounding, complete normalization of the BM with respect to the presence of an HS epitope recognized by the JM-13 MAb occurs much later, between 2 months and 1 year after injury. Since the JM-13 antibody recognizes a high-sulphated epitope of the HS side-chain, we speculated that the absence of a
fully sulphated HSPG might have an effect on functions associated with HSPG, such as charge-dependent permeability of the BM, assembly of BM components, and modulation of the binding of growth factors. We therefore investigated the kinetics of epidermal hyperproliferation up to 1 year after injury and we examined the presence of the high molecular weight plasma protein fibrinogen in the epidermal compartment. We found an association between the reappearance of the JM-13 epitope and the disappearance of a marker for epidermal proliferation (Ki-67). BM permeability was highest at day 14 as assessed by epidermal fibrinogen staining. After 2 months, fibrinogen staining was still increased but returned to normal patterns within 1 year, thus establishing another association in time, namely the presence of the JM-13 epitope in the BM and the absence of fibrinogen in the epidermis.

Healing of partial thickness wounds in normal human skin is characterized by a well-known sequence of events including inflammation, granulation,
fibroplasia and increased synthesis of ECM components, re-epithelialization, and finally remodelling of the scar tissue. Regeneration and synthesis of various ECM molecules during wound healing has been extensively studied.\textsuperscript{16,26-35} We have, for example, previously described changes of tenascin expression in the papillary dermis, during superficial skin injury\textsuperscript{16} and healing of partial thickness wounds using the same model as that in the present study.\textsuperscript{16} In addition, we recently investigated the proliferative behaviour of epidermal cells during normal and disturbed wound healing.\textsuperscript{24} To our knowledge, the dynamics of BM regeneration with respect to HSPG expression and the proliferative behaviour of the epidermis in the later phases of wound healing have not been described before.

Surprisingly, the epidermis remains hyperproliferative for a considerable time after wound closure. The morphology of the neo-epidermis during wound healing is quite distinct from the epidermis in other hyperproliferative conditions such as psoriasis, showing an absence of rete ridges and lack of acanthosis. The mechanism and function of the persistent epidermal hyperproliferation are unknown. The kinetics of normalization of epidermal hyperproliferation follow

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Fig. 3—Immunohistochemical staining for Ki-67 antigen, which is present in the nuclei of cycling cells (arrows) in normal human skin (A), in 60-day-old skin wounds (B), and 1 year after wounding (C). The number of cycling cells in the basal and first suprabasal layer is still increased at day 60 after wounding, but has returned to nearly normal values within 1 year after wounding.
for laminin and HSPG core protein and was found within 1 week after wounding. However, in previous investigations using formalin-fixed material rather than cryosections as in this study, the JW-403 epitope was found to be absent for up to 2 weeks. The discrepancy in staining intensity is probably caused by the low detection levels of most antigens on formalin-fixed, paraffin-embedded material. In the present study we used avidin-biotin complex enhanced staining on cryosections, which allows very sensitive detection of most antigens.

Other investigators have studied the expression of a number of different proteoglycans, including cell surface-associated HSPG, in the healing surface of mucosal wounds up to day 7. Using an antibody directed against the core protein of MAb HSPG is absent under migrating epithelial cells and the molecule is found to be restricted to endothelial keratocytes (CD44 and syndecan). These results and the data presented here suggest that HSPG, both cell membrane-bound and BM-associated, have multiple functions in the resurfacing of epithelial following injury.

REFERENCES


The kinetics of the normal epithelial BM with respect to the reappearance of the JW-13 epitope. Since a correlation does not necessarily imply a causal relationship, we have further studied the possible effect of high molecular weight proteins. Previous studies on high molecular weight proteins in experimental animals have shown that enzymatic removal of HS, neutralization of HS charge by ion exchange or injection of a MAbspecific for GBM, HS, transiently leads to an ablation in the situation in the glomerulus, a decrease in HSPG amount in the dero-membrane BM. Assuming the presence of epitope. In the absence of the JW-13 epitope in the dero-epithelial BM, could allow cell passage of mitogenic proteins into the epithelial compartment, thus leading to a change in the basement membrane. An alternative explanation for the presence of the O-sulfated HSPG epitopes is the absence of the basement membrane, which could alter the growth behaviour of the basal lamina. However, since we have no support for evidence with respect to growth factor binding during wound healing, this contention remains speculative. We clearly show that positive subendothelial BM staining is variable and difficult to quantify since only a limited number of biopsies were studied, and we cannot draw firm conclusions on the basis of these results.


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