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Time-dependent pericellular expression of collagen type IV, laminin, and heparan sulfate proteoglycan in myofibroblasts

P. Betz[1], A. Nerlich[2], J. Wilske[1], J. Tübel[1], I. Wiest[2], R. Penning[1], and W. Eisenmenger[1]

Department of Legal Medicine[1] and Pathology[2], University of Munich, Frauenlobstrasse 7a, W-8000 München, Federal Republic of Germany

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Summary. Human skin wounds ($n = 62$) with a wound age between 5 h and 6 weeks were investigated. The appearance of cell-associated pericellular basement membrane components collagen type IV, laminin and heparan sulfate proteoglycan (HSPG) in myofibroblasts was evaluated by immunohistochemistry. Laminin and HSPG were first detectable around myofibroblasts approximately 1.5 days after wounding. Collagen type IV did not appear before the 4th day after wound infliction. In wounds more than 7 days old, 94% of the cases showed fibroblastic cells positively staining for laminin, 70% of the wounds contained fibroblastic cells positive for HSPG and in 63% a positive reaction for collagen type IV was obtained around these cells. The numbers of the cases as well as of the cells positively stained for laminin exceeded the corresponding values for HSPG and especially for collagen type IV. The pericellular appearance of laminin or HSPG around myofibroblasts, therefore, indicates a wound age of at least approximately 1.5 days. The pericellular localization of collagen type IV indicates a survival time of approximately 4 days or more. Since these proteins are still detectable in the pericellular region of myofibroblasts in skin wounds with advanced wound age (6 weeks) further information for the time-estimation of older human skin lesions cannot be obtained. A semiquantitative analysis revealed no significant correlation between the number of positively stained cells and the wound age, rendering this parameter unsuitable for a practicable time-estimation of human wounds.

Key words: Collagen type IV – Laminin – Heparan sulfate proteoglycan – Wound age – Immunohistochemistry

Zusammenfassung. An insgesamt 62 menschlichen Hautwunden mit einer Überlebenszeit zwischen 5 Stunden und 6 Wochen wurde das wundaltersabhängige perizelluläre Auftreten der Basalmembran-Komponenten Kollagen Typ IV, Laminin und Heparansulfat-Proteoglycan (HSPG) in Myofibroblasten untersucht. Laminin und HSPG waren erstmals in einer 1,5 Tage überlebten Hautwunde nachweisbar, Kollagen IV konnte erst nach ca. 4 Tagen beobachtet werden. In Hautwunden mit einem Wundalter von 1 Woche und mehr konnte in 94% der Fälle Laminin, in 70% HSPG und in 63% Kollagen IV perizellulär nachgewiesen werden. Laminin trat hierbei nicht nur in einem höheren Prozentsatz der Fälle, sondern auch in einer größeren Anzahl von Myofibroblasten im Vergleich zu HSPG und v.a. zu Kollagen IV auf. Der positive Nachweis von Laminin oder HSPG bzw. von Kollagen IV in der perizellulären Region von Myofibroblasten weist somit auf ein Wundalter von mindestens ca. 1,5 bzw. 4 Tagen hin. Da die untersuchten Basalmembran-Komponenten auch noch um Myofibroblasten älterer Hautwunden (6 Wochen Wundalter) nachweisbar waren, kann durch die immunhistochemische Darstellung dieser Proteine keine zusätzliche Aussage über das Alter von Wunden mit längerer Überlebenszeit getroffen werden. Die semiquantitative Auswertung ergab keine für eine Wundaltersbestimmung verwertbare Korrelation zwischen der Zahl positiv anfärbbarer Myofibroblasten und der Überlebenszeit.

Schlüsselwörter: Kollagen IV – Laminin – Heparansulfat-Proteoglycan – Wundalter – Immunhistochemie

Introduction

Basement membranes separate parenchymal cells from their surrounding connective tissue and are composed of several specific proteins such as collagen IV, laminin or the basement membrane heparan sulfate proteoglycan (HSPG) [8, 10, 11].

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Correspondence to: P. Betz

These basement membrane components are synthesized by the adjacent cells such as epithelial or endothelial cells. Recently, it was shown that myofibroblasts are surrounded by a basement membrane. This specialized cell type preferentially appears during wound healing [13].

The present immunohistochemical study was performed to define whether the immunohistochemical localization of the basement membrane components collagen type IV, laminin, and HSPG in myofibroblasts may present reliable parameters for a time-estimation of human skin wounds.

Materials and methods

In 62 human skin wounds (surgical wounds, lacerations and stab wounds after surgical treatment) with a wound age between 5h and 6 weeks, collagen IV and laminin were localized immunohistochemically. The localization of HSPG was visualized in 36 of these cases.

The specimens were obtained and prepared as previously described [4, 5].

After enzyme pretreatment (pepsin) paraffin sections were stained using polyclonal antibodies against collagen type IV and laminin (Eurodiagnostics, Leiden, Netherlands). In 36 skin wounds a polyclonal antibody against porcine HSPG has also been applied (kindly supplied by Dr. E. Schleicher, Institute for Diabetes Research, Munich, Germany) according to the ABC-method [9].

Uninjured skin from the same patients as well as sections stained without primary antibody served as controls.

A semiquantitative analysis using a 3-grade scoring-system was performed. The score ranged from 0 (no positively reacting cells) to 3 (numerous reacting cells in the wound area). Only those spindle-shaped cells which showed no topographical relation to endothelial cells of newly forming capillaries in the lesional area were regarded as positively reacting myofibroblasts.

Results

Uninjured skin

The basement membrane of the epidermal layer as well as the basement membrane of skin appendages and blood vessel stained positively for collagen type IV, laminin, and HSPG. Around nerve cells (Schwann cells, perineural cells) and muscle fibers a positive reaction was also found. The basal lamina of lymph capillaries and the basement membrane surrounding adipocytes reacted with collagen IV and laminin, but not with HSPG.

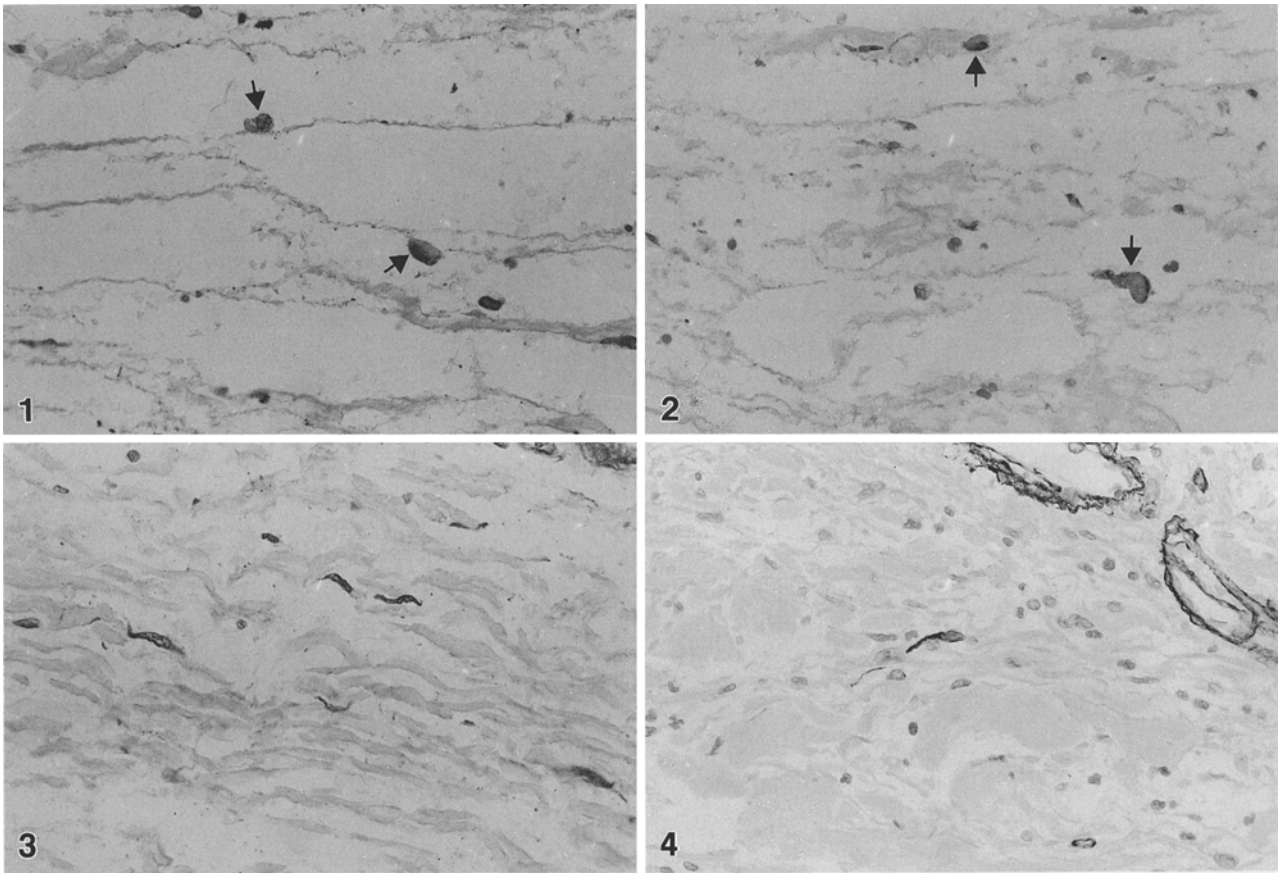

Fig. 1. Myofibroblasts positively stained for heparan sulfate proteoglycan in a 2-day-old human skin wound (paraffin, ABC-method, 480 ×)

Fig. 2. Positive reaction for laminin around myofibroblasts in a skin wound aged 2 days (paraffin, ABC-method, 480 ×)

Fig. 3. Skin wound with a wound age of 11 days: myofibroblasts showing positive staining for laminin (paraffin, ABC-method, 300 ×)

Fig. 4. Single myofibroblast positively stained for collagen IV in a 4-week-old skin wound (paraffin, ABC-method, 480 ×)

Skin wounds

Collagen type IV. A cell-associated deposition of collagen IV around myofibroblasts could be detected in a 4-day-old wound but not in wounds of a lesser duration from infliction. In skin wounds with a wound age between 4 and 11 days ($n = 23$) only 3 cases (13%) showed positively staining myofibroblasts in minor amounts (grade 1). From the 12th day from infliction up to 6 weeks ($n = 25$) the staining was variable. Specimens without positive cells as well as with numerous positively staining myofibroblasts were seen (6 cases ranked as grade 0 (24%), 14 cases as grade 1 (56%), 2 cases as grade 2 (8%) and 3 cases as grade 3 (12%). Myofibroblasts positive for collagen IV were also present in the oldest wound investigated (wound age 6 weeks). In 5 cases a network of collagen type IV fibers had developed around the positively reacting myofibroblasts. The wound age of these specimens ranged between 14 days and 6 weeks.

Laminin. The earliest appearance of laminin-producing myofibroblasts was observed in a specimen with a wound age of 1.5 days. Between 1.5 and 5 days, 8 out of 17 cases (44%) revealed negative results, while the other specimens showed variable values (ranging from grade 1 (28%) up to grade 2 (29%)). In only 2 out of 35 cases with a wound age of more than 7 days (6%) no laminin-positive myofibroblasts could be identified. The other skin wounds aged between 7 days and 6 weeks showed between few (12 cases (37%) belonging to grade 1) and numerous (8 cases (29%) belonging to grade 3) positive cells. Network-like structures positive for laminin occurred around the myofibroblasts in only 2 specimens (wound age 26 days or 4 weeks).

Heparan sulfate proteoglycan. Analogous to the results for laminin, HSPG was first detectable in a skin wound of 1.5 days duration. Compared to laminin the number of positively reacting myofibroblasts was somewhat lower. Out of 27 cases with a wound age between 1.5 days up to 6 weeks, 10 skin wounds showed no positive cells (37%), 11 lesions were ranked to grade 1 (41%), 5 cases belonged to grade 2 (18%), the remaining case (4%) to grade 3. Extracellular network-like structures of HSPG, could be found in the skin wound (duration 4 weeks) in which a network of laminin fibers had also been observed, but not in any other specimens. This basement membrane component was also detectable – like collagen IV and laminin – in the oldest wound (wound age 6 weeks) investigated.

There were no identifiable relevant differences which could relate the earliest appearance of collagen IV, laminin, and HSPG to the donor's age or to the site of localization of the wound.

Discussion

The most useful methods for the age determination of skin wounds with a survival time of a few days are the detection of hemosiderin [2, 3, 12] and the immunohistochemical localization of different collagen subtypes [7]. Furthermore, the time-dependent rearrangement of the epithelial basement membrane by immunohistochemical detection of basement membrane components can provide information for a forensically applicable time-estimation of human skin wounds [4, 7]. During wound healing, basement membrane material also occurs around proliferating "myofibroblasts" as demonstrated by ultrastructural studies [1, 11]. Previous reports suggest that an immunohistochemical analysis of basement membranes may be significant for time-dependent changes in basement membrane components in various pathological conditions [13].

During wound healing fibroblasts can undergo transformation to so-called "myofibroblasts", a subspecies of fibroblastic cells characterized by the content of various cytoplasmic filaments such as vimentin, alpha smooth muscle actin, and desmin [14]. Besides the transient expression of alpha smooth muscle actin [5, 6], myofibroblasts are able to synthesize various extracellular matrix components during wound healing such as the basement membrane components collagen type IV, laminin, and HSPG [13]. Studies dealing with the time-dependent appearance of collagen IV, laminin, and HSPG around myofibroblasts of skin wounds, especially in human skin wounds, have not been performed previously.

In our series, myofibroblasts positive from laminin and HSPG could be detected first in a wound aged 1.5 days. Myofibroblasts positive for collagen IV, however, were demonstrable first in a 4-day-old wound. Collagen type IV did not only appear later, but also in lower amounts and in a considerably smaller number of cases when compared to the demonstrability of HSPG and especially to laminin. With very few exceptions (only 2 out of 35 cases with a wound age of more than 5 days showed no positive myofibroblasts), laminin was regularly demonstrable around myofibroblasts of the skin wounds investigated. The earlier and more regular appearance of laminin – and to lesser extent of HSPG – when compared to collagen IV is possibly explained by the different biological functions of these proteins. Collagen type IV is mainly responsible for mechanical stability by aiding the development of a 3-dimensional network [10]. The role of collagen IV in cell-cell-interaction as compared to that of laminin and HSPG [8] appears to be of less importance. This might explain the observed differential appearance of the basement membrane components around myofibroblasts while the appearance of basement membrane components during the rearrangement of the epithelial basement membrane shows no significant age-related differences [7].

In our series, collagen IV, laminin, and HSPG were also detectable in the wound with the longest post-infliction period (wound age: 6 weeks) indicating that the immunohistochemical localization of these basement membrane components provides no useful additional information on wound timing.

The semiquantitative evaluation of the number of myofibroblasts positively stained for collagen type IV, laminin or HSPG revealed a considerable variability and showed no clear correlation to wound age. No reliable or

useful information for the time-estimation of human skin wounds, can be obtained by a numerical assessment of myofibroblasts positively staining for basement membrane components because negative results can also occur.

Conclusion

The immunohistochemical detection of the basement membrane components collagen type IV, laminin, and HSPG allows the following statements regarding a forensically applicable time-estimation of human skin wounds:

1. The pericellular appearance of laminin and HSPG around myofibroblasts indicates a wound age of at least approximately 1.5 days. Laminin seems to be expressed in a larger number of cells and wounds as compared to HSPG rendering the immunohistochemical localization of laminin a more useful parameter in this context.
2. Pericellular collagen type IV indicates a survival time of at least approximately 4 days and appears considerable later than laminin or HSPG.
3. Collagen type IV, laminin, and HSPG are also expressed in myofibroblasts of skin wounds with an advanced post-infliction duration providing no further useful information for the age-determination of older skin lesions.
4. Cases without positively reacting myofibroblasts were also observed indicating that the lack of positive staining in myofibroblasts cannot exclude a wound age of more than 1.5 or 4 days.

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