0022-202X/81/7702-0240$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 77:240-243, 1981

Vol. 77, No. 2
Printed in U.S.A.

Detection of Basement Membrane Zone Antigens During Epidermal Wound Healing in Pigs

JOHN R. STANLEY, M.D., OSCAR M. ALVAREZ, PH.D., E. WILLIAM BERE, JR., WILLIAM H. EAGLSTEIN, M.D., STEPHEN I. KATZ, M.D., PH.D.

Dermatology Branch of the National Cancer Institute (JRS, EWB, SIK) and the Laboratory of Developmental Biology and Anomalies of the National Institute of Dental Research (JRS), National Institutes of Health, Bethesda, Maryland, and the Department of Dermatology, University of Miami, (OMA, WHE), Miami, Florida, U.S.A.

Bullous pemphigoid (BP) antigen, laminin, and type IV collagen, 3 distinct antigens of basement membrane, were studied by indirect immunofluorescence in the epidermal-dermal junction of re-epithelializing wounds. Partial thickness wounds were made with a dermatome in the skin of white Yorkshire pigs. After 2 or 3 days, the wound site and the surrounding normal skin were excised and cryostat sections were studied using BP sera as well as whole antisera and affinity purified antibodies to laminin and type IV collagen. Laminin and type IV collagen were detected in the basement membrane zone of normal epidermis and at the re-epithelializing epidermal-dermal junction for a variable distance into the healing wound but both were absent from the more distal migrating epidermis. In contrast, BP antigen extended from the basement membrane zone of normal skin throughout the entire epidermal-dermal junction of the wound as far as the distal tip of the migrating epidermis. These results suggest that in the re-epithelization of superficial wounds laminin and type IV collagen are not present in the initial epidermal-dermal interaction of the migrating epithelium but that BP antigen may be important in this early interaction.

The basement membrane zone (BMZ) of epidermis has been shown to contain several distinct antigens. By indirect immunofluorescence bullous pemphigoid (BP) antigen has been localized to the BMZ of stratified squamous epithelia [1,2] but not other basement membranes. Ultrastructurally, BP antigen has been localized to the lamina lucida [3,4], the electron lucent region just below the basal cell plasma membrane, and is probably closely associated with the basal cell plasma membrane [5,6]. Laminin is a noncollagenous protein of basement membrane, and unlike BP antigen, is found in all basement membranes [7]. Thus by indirect immunofluorescence of skin, laminin is found not only in the epidermal BMZ but also around blood vessels and smooth muscle [7]. Ultrastructurally, laminin is localized in the lamina lucida [7]. Finally, type IV collagen is also present in all basement membranes and by indirect immunofluorescence has the same distribution as laminin [8]. However, ultrastructurally type IV collagen is localized to the basal lamina [8], the electron dense band which is below the lamina lucida. Thus in normal epidermis BP antigen, laminin, and type IV collagen are all expressed in the intact epidermal BMZ.

Manuscript received October 28, 1980; accepted for publication January 26, 1981.

Dr. Stanley is an Intergovernmental Personnel Act (IPA) visiting scientist at the National Cancer Institute from the Department of Dermatology, New York University Medical Center, New York, NY.

Reprint requests to: Dr. John R. Stanley, Building 10, Room 12N238, National Institutes of Health, Bethesda, Maryland 20205.

Abbreviations:
BMZ: basement membrane zone
BP: bullous pemphigoid

In re-epithelializing wounds the normal ultrastructure of the BMZ is lost below the migrating epidermal cells [9-11]. The question of which, if any, of these antigens is present under the migrating epidermis has not been addressed. However, migrating epidermis *in vitro* has been studied. BP antigen is present under migrating epidermis during the process of epiboly in which epidermis migrates around the cut edge of the dermis in organ culture [12-14]. This antigen is also present under epidermal cells growing on dead pig skin [15], on an inert collagen substrate [16] and on glass [17]. Hintner et al [14] have recently shown by immunofluorescence that, during the process of epiboly, BP antigen is present under all the migrating epidermis including the advancing tip, whereas laminin and type IV collagen appear under the migrating epidermis only after a delay of several days and never appear under the advancing tip of the migrating epidermis. Stenn, Madri, and Roll [18] also studied epiboly as well as epidermal migration on Millipore filters, and found that in the first 2 to 3 days of culture the migrating epidermis lacked type IV collagen. In addition, recent reports suggest that although BP antigen is present under epidermis migrating on dead dermis [15], type IV collagen is not [19]. Thus, *in vitro*, BP antigen appears to be important early in migrating epidermis-substrate interactions, whereas the appearance of laminin and type IV collagen is delayed.

To determine whether these observations are also true *in vivo*, we used a recently developed model of wound healing [20] in order to determine which of these BMZ antigens was present under the migrating epidermis during re-epithelialization.

MATERIALS AND METHODS

Wounding Method

As previously reported [20] standard wounds 7 mm × 10 mm and 0.3 mm deep were made in the paravertebral and thoracic areas of 5 to 9 kg white Yorkshire pigs using a Castroviejo electrokeratome (Storz Surgical Instruments, St. Louis, MO). Two or 3 days later the wounds were harvested using the keratome with a 15-mm blade and set to a depth of 0.7 mm. Each specimen included the wound and adjacent unwounded skin. The day of wounding was designated day 0 and six, 3-day wounds and two 2-day wounds were studied.

Antisera and Antibodies

Sera from 6 patients with BP were used to identify BP antigen in the healing wound. Five of these sera were titered by immunofluorescence using normal pig and/or normal human skin, and reacted with the epidermal BMZ at titers of greater than 1:160. For studying wounds, BP sera were generally used at dilutions of 1:10 or 1:20 to ensure antibody excess. At these dilutions all sera reacted strongly with the BMZ of unwounded pig skin. Each serum gave identical results.

Rabbit antisera to laminin and type IV collagen were prepared as previously reported [7,8]. These antisera reacted with the epidermal BMZ of normal pig skin as well as with blood vessel basement membranes at immunofluorescent titers of greater than 1:320. For studying wounds sera were used at dilutions of 1:10 to 1:80 to ensure antibody excess. In addition, affinity purified antibodies to laminin and type IV collagen were prepared. The antigens, laminin, and type IV collagen, were purified from the EHS mouse sarcoma [21-23] and were kindly provided by Dr. J.M. Foidart. Sheep were immunized by subcutaneous injections of 1 mg of protein in phosphate buffered saline after emulsion

with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan). Two boosters of 0.5 mg of antigen each, emulsified in an equal volume of incomplete Freund's adjuvant, were given at 2 week intervals. The sheep were plasmapheresed 2 to 4 weeks later. Antibodies to laminin were purified from plasma by cross-immunoabsorption on a type IV collagen-Sepharose 4B column, after which the plasma was absorbed on a laminin-Sepharose 4B column from which the antibodies were eluted [7]. Similarly, antibodies to type IV collagen were first run over a laminin-Sepharose 4B column, then absorbed on a type IV collagen-Sepharose 4B column from which they were eluted. The specificity of the antibodies was confirmed using immunoelectron microscopy [7,8] which showed that laminin antibodies bound to the lamina lucida of normal human skin BMZ whereas type IV collagen antibodies bound to the basal lamina.

By indirect immunofluorescence, antibodies were detected bound to basement membranes of normal pig skin when laminin or type IV collagen antibodies were used at concentrations as low as 25 to 30 μg/ml. To study wounds, antibodies were used at concentrations at 50 to 200 μg/ml. Antisera and purified antibodies gave identical results regardless of dilution.

Indirect Immunofluorescence

Wounds, which included unwounded adjacent skin, were embedded in Tissue-Tek II O.C.T. (Lab Tek Products, Naperville, IL) and frozen. Sections of 6 μm were cut in a cryostat and placed on albuminized slides. Indirect immunofluorescence was performed as previously described [1,17]. The first serum or antibody applied was BP serum, normal human serum, rabbit antiserum or sheep antibodies to laminin or type IV collagen, normal rabbit serum or normal sheep IgG. The second antibody applied was the appropriate fluorescein-isothiocyanate-conjugated antibody: goat anti-human IgG, goat anti-rabbit IgG or rabbit anti-sheep IgG (Cappel Laboratories, Cochranville, PA).

RESULTS

We chose to study day 2 and day 3 wounds because these provided a full spectrum of the stages of re-epithelialization of the wound. Most day 3 and both day 2 wounds were not completely re-epithelialized and, therefore, different parts of the wounds were in different stages of the healing process. Each wound was sectioned so that the normal skin adjacent to the wound as well as the wound could be visualized microscopically in one section. The wound epithelium could easily be identified microscopically because it was seen under a crust and because it lacked rete ridges. The wound epithelium became progressively less stratified as it extended further into the wound so that the distal tip of the wound epithelium was only 1 or 2 cell layers. Beyond this was the nonepithelialized wound where the crust abutted directly on the cut surface of the dermis. In addition, we studied a completely re-epithelialized section of a day 3 wound.

The basement membrane antigens BP, laminin, and type IV collagen were detected by immunofluorescence. In normal skin adjacent to the wound, laminin and type IV collagen were seen both at the epidermal BMZ and around blood vessels (Fig 1*A*). These 2 antigens extended for variable distances into the wound at the epidermal-dermal junction, but never extended as far as the distal tip of the migrating epidermis (Fig 1*B*, 2*C*, 3*B*). The transition between areas in which antigen was detectable and undetectable was abrupt (Fig 2*C*, 3*B*). In one characteristic day 3 wound, the wound epithelium measured 300 μm whereas laminin and type IV collagen extended only 50 μm into the wound from the adjacent normal skin. In contrast to these findings at the epidermal-dermal junction, laminin and type IV collagen were detected around blood vessels in all parts of the wound. In one completely re-epithelialized section of a day 3 wound, laminin and type IV collagen were present along the entire BMZ. Considering all the wounds examined, we detected no consistent differences in the distribution of laminin compared to type IV collagen. Taken altogether, these findings suggest that in the migrating epidermis of re-epithelializing wounds laminin and type IV collagen do not appear concordant with the epidermal-dermal contact but appear only after a delay. Alternatively, it is possible, but unlikely, that in the

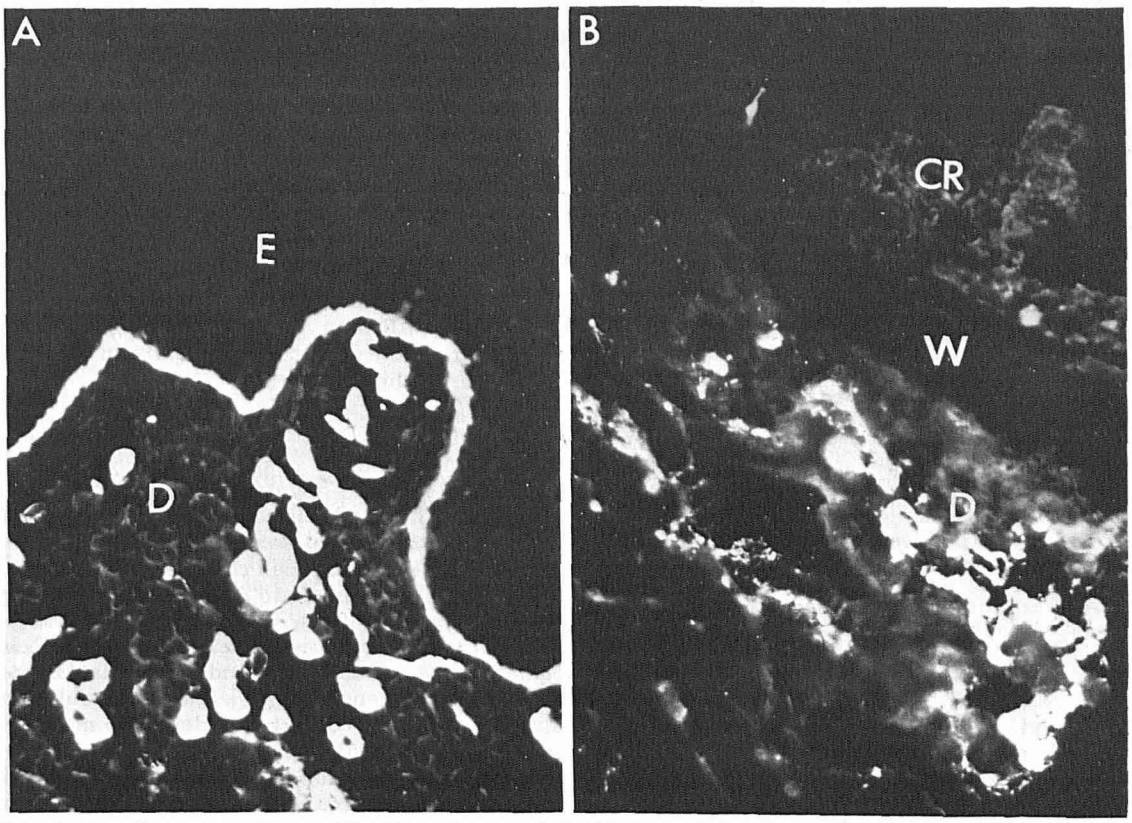

FIG 1. Indirect immunofluorescence of type IV collagen in normal pig skin and adjacent day 2 wound. *A*, Normal skin. Basement membranes of epidermis and blood vessels fluoresce (× 250). *B*, Re-epithelializing wound. Type IV collagen is seen around blood vessels but not at the epidermal-dermal junction (× 250). *E*, normal epidermis; *W*, wound epidermis; *CR*, crust, *D*, dermis.

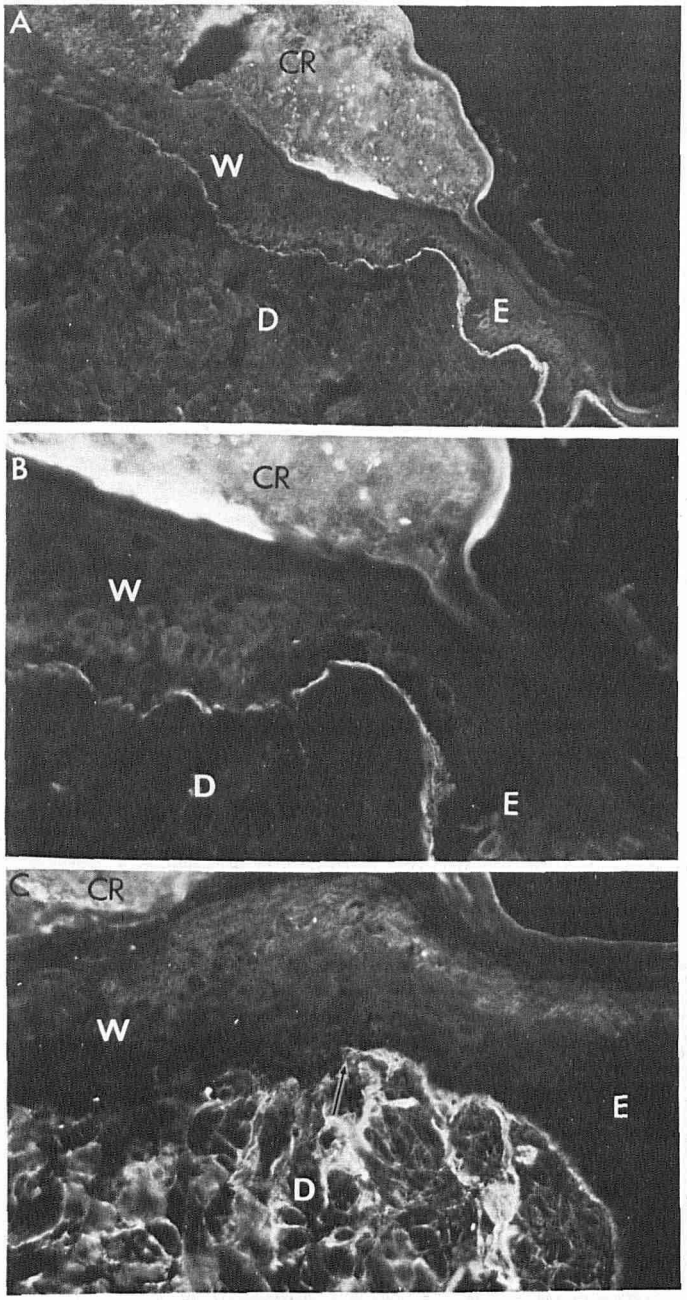

FIG 2. Indirect immunofluorescence of BP antigen and type IV collagen in a day 2 wound. *A*, BP antigen extends from normal skin throughout entire wound at the epidermal-dermal junction (reduced from × 100). *B*, Higher magnification of 2*A*. BP antigen is continuous from normal skin to the epidermal-dermal junction of the wound (reduced from × 260). *C*, Type IV collagen at the epidermal-dermal junction extends only a short distance into the wound. The transition between detectable and undetectable antigen is abrupt (*arrow*). There is some nonspecific background fluorescence of the dermis in this section. *E*, normal epidermis; *W*, wound epidermis; *CR*, crust; *D*, dermis.

distal wound these 2 antigens are present but masked. This is especially unlikely because the blood vessels in the wound display these antigens (Fig 1*B*, 3*B*) and because the transition between detectable and undetectable antigen is abrupt.

In contrast to laminin and type IV collagen, BP antigen was detected as a band of fluorescence extending from the BMZ of normal epidermis throughout the entire epidermal-dermal junction of the wound (Fig 2*A–B*, 3*A*), including the distal end of the migrating epithelial sheet. The intensity of fluorescence was diminished in the wound BMZ, and at the distal tip of the

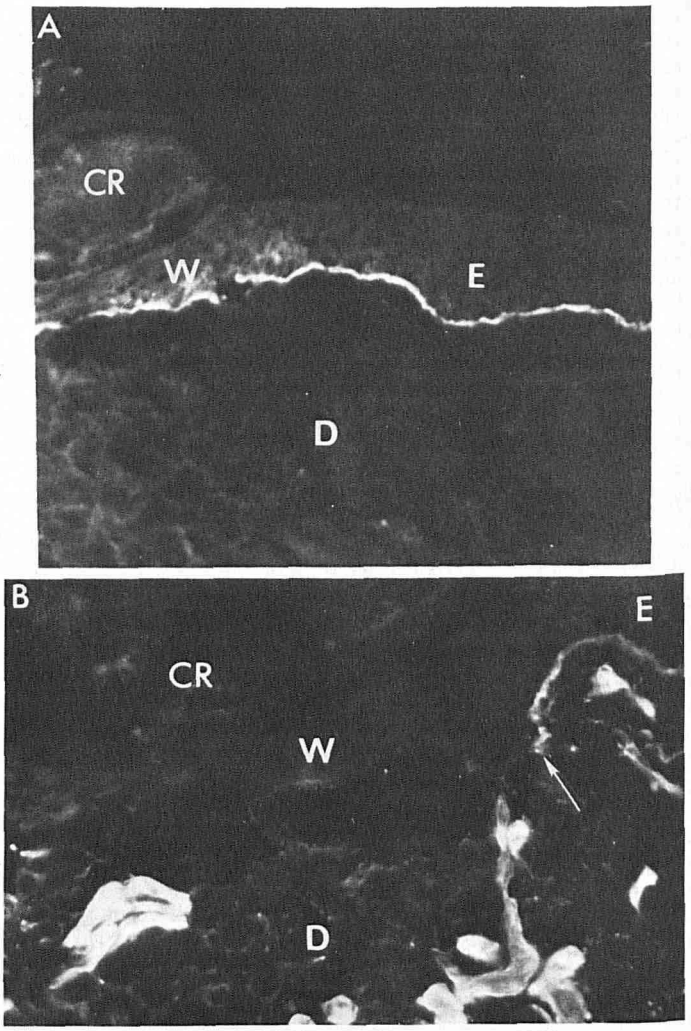

FIG 3. Indirect immunofluorescence of BP antigen and laminin in a day 3 wound. *A*, Bullous pemphigoid antigen extends throughout wound (reduced from × 125). *B*, Laminin at wound epidermal-dermal junction stops abruptly (*arrow*), however, it is present around blood vessels throughout the wound (reduced from × 250). *E*, normal epidermis; *W*, wound epidermis; *CR*, crust, *D*, dermis.

migrating epidermis the fluorescence sometimes became patchy, but in all cases the antigen was detected throughout the wound epithelium. These results suggest that BP antigen is present from the outset or very early in epithelial-substrate contact in the healing wound.

DISCUSSION

Epidermal basal cell contact with an appropriate substrate may be essential in maintaining the integrity of the normal epidermis. The basal cell, compared to the more differentiated cells of the epidermis, has a special affinity for substrates, including type IV and type I collagen [6]. The contact between the basal cell and substrate may be important in maintaining the proliferative ability of the basal cell as well as in preventing its terminal differentiation. For example, cells of chick embryo epidermis cease proliferating and differentiate in the absence of contact with mesenchyme [24]. Similarly, adult human epidermis requires direct contact with dermis to maintain a proliferating basal layer [25]. Separation of epidermis and dermis by a Millipore filter prevents proliferation. Finally, epidermal cells in suspension culture, separated from mesenchymal contact, rapidly differentiate and lose the ability to proliferate when replated on 3T3 (a fibroblast cell line) feeder layers in tissue culture [26,27].

In normal epidermis the basal cell contacts the dermal substrate at the basement membrane zone (BMZ). Several antigens, including BP antigen, laminin and type IV collagen, have been localized to this zone, and certain of these may be important in this basal cell-substrate interaction. For example, laminin may act as an attachment factor between basal cells and the type IV collagen of the basal lamina [28]. In a re-epithelializing wound, on the other hand, the basal cell leaves the BMZ and migrates directly over the dermal wound bed [9–11]. Ultrastructurally, there is no basal lamina or lamina lucida under these migrating cells. In this specific basal cell-substrate interaction the cell must adhere to the wound bed and maintain its ability to proliferate just as in normal epidermis. In addition the cell must be able to migrate on this substrate. In this report, we determined whether 3 antigens found in the normal epidermal BMZ were also present in the wound epidermal-substrate junction.

Our finding that epidermal cells were not seen in contact with the substrate in the absence of BP antigen suggests that BP antigen may have an important role in the early basal cell-substrate interaction of these healing wounds. In contrast, laminin and type IV collagen were not detectable in the leading edge of the migrating epidermis and only appeared later when the wound was more fully healed. These findings are in agreement with studies done *in vitro* which show that epidermal cells migrating over dermis display BP antigen at the cell-substrate interface [12–15] but may lack laminin and type IV collagen [14,18,19], especially at the leading edge of the migrating cells.

Thus, before the epidermis has a fully established basement membrane zone, BP antigen may play a role in the basal cell-substrate interaction. Its exact role has not been determined but it might be important in basal cell-substrate adhesion and migration, in preventing terminal differentiation and maintaining proliferation of the basal cells, or as a signal for production of other basement membrane zone components. *In vitro*, AB_2 collagen has also been found to be present early in the migrating epidermal-substrate junction [18]. It has not been determined whether this collagen is also detectable in migrating epidermis *in vivo*.

Although the distal areas of the wound epidermis lacked laminin and type IV collagen, the proximal, more stratified areas, displayed these antigens, and a fully re-epithelialized wound displayed them across the entire healed epidermal-dermal junction. These results suggest that laminin and type IV collagen appear rapidly as the epidermis re-establishes its normal configuration. These 2 antigens, therefore, may be important in maintaining the integrity of normal differentiating and nonmigrating epidermis.

REFERENCES

1. Beutner EH, Chorzelski TP, Bean SF, Jordon RE: Immunopathology of the Skin Labeled Antibody Studies. Dowden, Hutchinson, & Ross, Inc., Stroudsburg, PA, 1973
2. Diaz LA, Weiss HJ, Calvanico NJ: Phylogenetic studies with pemphigus and pemphigoid antibodies. Acta Dermatovener (Stockh) 58:537–540, 1978
3. Schaumburg-Lever G, Rule A, Schmidt-Ullrich B, Lever WF: Ultrastructural localization of in vivo bound immunoglobulins in bullous pemphigoid—A preliminary report. J Invest Dermatol 64:47–49, 1975
4. Holubar K, Wolff K, Konrad K, Beutner EH: Ultrastructural localization of immunoglobulins in bullous pemphigoid skin. J Invest Dermatol 64:220–227, 1975
5. Masutani M, Ogawa H, Taneda A, Shoji M, Miyazaki H: Ultrastructural localization of immunoglobulins in the dermo-epidermal junction of patient with bullous pemphigoid. J Dermatol 5:107–112, 1978
6. Stanley JR, Foidart JM, Murray JC, Martin GR, Katz SI: The epidermal cell which selectively adheres to a collagen substrate is the basal cell. J Invest Dermatol 74:54–58, 1980
7. Foidart JM, Bere EW, Yaar M, Rennard SI, Gullino M, Martin GR, Katz SI: Distribution and immunoelectron microscopic localization of laminin, a noncollagenous basement membrane glycoprotein. Lab Invest 42:336–342, 1980
8. Yaoita H, Foidart JM, Katz SI: Localization of the collagenous component in skin basement membrane. J Invest Dermatol 70: 191–193, 1978
9. Odland G, Ross R: Human wound repair. I. Epidermal regeneration. J Cell Biol 39:135–151, 1968
10. Krawczyk WS: Some ultrastructural aspects of epidermal repair in two model wound healing systems. Epidermal Wound Healing. Edited by HI Maibach and DT Rovee. Chicago, Year Book Medical Publishers, 1972, pp 123–131
11. Martinez R: Fine structural studies of migrating epithelial cells following incisional wounds. Epidermal Wound Healing. Edited by HI Maibach and DT Rovee. Chicago, Year Book Medical Publishers, 1972, pp 323–342
12. Marks R, Abell E, Nishikawa T: The junctional zone beneath migrating epidermis. Br J Dermatol 92:311–319, 1975
13. Woodley D, Regnier M: In vitro bullous pemphigoid antigen deposited on a Millipore filter. Arch Dermatol Res 266:319–322, 1979
14. Hintner H, Fritsch PO, Foidart JM, Stingl G, Schuler G, Katz SI: Expressions of basement membrane zone antigens at the dermo-epibolic junction in organ cultures of human skin. J Invest Dermatol 74:200–204, 1980
15. Woodley D, Vidiergean L, Regnier M, Saurat J, Prunieras M: Bullous pemphigoid antigen synthesized in vitro by human epidermal cells, J Invest Dermatol 75:148–151, 1980
16. Stanley JR, Hawley-Nelson P, Poirier M, Katz SI, Yuspa SH: Detection of pemphigoid antigen, pemphigus antigen, and keratin filaments by indirect immunofluorescence in cultured human epidermal cells. J Invest Dermatol 75:183–186, 1980
17. Yuspa SH, Hawley-Nelson P, Koehler B, Stanley JR: A survey of transformation markers in differentiating epidermal cell lines in culture. Cancer Res, 40:4694–4703, 1980
18. Stenn KS, Madri JA, Roll FJ: Migrating epidermis produces AB_2 collagen and requires continual collagen synthesis for movement. Nature 277:229–232, 1979
19. Woodley DT, Regnier M, Prunieras M: Basal lamina formation may require dermal or serum factors (abstract). J Invest Dermatol 74:248–249, 1980
20. Eaglstein WH, Mertz PM: New method for assessing epidermal wound healing. The effects of triamcinolone acetonide and polyethelene film occlusion. J Invest Dermatol 71:282–284, 1978
21. Orkin RW, Gehron P, McGoodwin EB, Martin GR, Valentine T, Swarm R: A murine tumor producing a matrix of basement membrane. J Exp Med 145:209–219, 1977
22. Timpl R, Martin GR, Bruckner P, Wick G, Wiedemon H: Nature of the collagenous protein in a tumor basement membrane. Eur J Biochem 84:43–52, 1978
23. Timpl R, Rohre H, Robey PG, Rennard SI, Foidart JM, Martin GR: Laminin—A glycoprotein from basement membranes. J Biol Chem 254:9933–9937, 1979
24. McLoughlin CB: The importance of mesenchymal factors in the differentiation of chick epidermis. J Embryol Exp Morph 9:370–409, 1961
25. Briggaman RA, Wheeler CE: Epidermal-dermal interactions in adult human skin: Role of dermis in epidermal maintenance. J Invest Dermatol 51:454–465, 1968
26. Green H: Terminal differentiation of cultured human epidermal cells. Cell 11:405–416, 1977
27. Morrissey JH, Green H: Differentiation-related death of an established keratinocyte line in suspension culture. J Cell Physiol 97: 469–476, 1978
28. Terranova VP, Rohrbach DH, Martin GR: Role of laminin in the attachment of PAM 212 epithelial cells to basement membrane collagen. Cell, 22:719–726, 1980