Defective Expression of Basement Membrane-Associated C3d,g in Papulonodular Basal Cell Carcinomas

Nicole Basset-Séguin, M.D., Paul Uhle, M.D., Diane Emanuel, B.S., Paula Henry, M.A., and Kim B. Yancey, M.D.

Department of Dermatology, Uniformed Services University of the Health Sciences, Bethesda, Maryland (NB-S, PH, KBY); Department of Dermatology, Hospital Saint-Charles, Montpellier, France (NB-S); Department of Dermatology, Naval Medical Center, National Capitol Region, Bethesda, Maryland (PU); and School of Medicine, Georgetown University, Washington, D.C., U.S.A. (DE)

Recent studies in our laboratory have shown that C3d,g, a 41,000-Da fragment of the third component of complement, is present along the base of the lamina densa and in the sublamina densa region of normal human epidermal basement membrane, but absent from the skin of a patient with congenital C3 deficiency. In studies of human skin, papulonodular basal cell carcinomas have served as a useful model for the investigation of various basement membrane antigens and matrix proteins. To further investigate the presence of C3d,g within epidermal basement membrane as well as examine its relationship with other known basement membrane constituents, we have analyzed serial sections of ten papulonodular basal cell carcinomas by light and immunofluorescence microscopy. In these studies, C3d,g was either absent (N = 9) or minimumly detectable (N = 1) in tumor nest basement membranes. While bullous pemphigoid and KF-1 antigens were absent (N = 6 and N = 3, respectively) or significantly decreased (N = 4 and N = 7, respectively), epidermolysis bullosa acquisita antigen was routinely present though somewhat (N = 3) or moderately decreased (N = 3). Laminin and type IV collagen were expressed normally in all tumor nest basement membranes. All constituents, including C3d,g, were present in adjacent normal epidermal basement membrane of these tumor samples. This study has demonstrated antigenic alterations within each ultrastructural subregion of papulonodular basal cell carcinoma tumor nest basement membranes by identifying the virtual absence of C3d,g (sublamina densa) as well as a significant reduction in KF-1 (lamina densa) and bullous pemphigoid (lamina lucida) antigens. Moreover, the presence of laminin, type IV collagen, and epidermolysis bullosa acquisita antigen in tumor nest basement membranes suggests that these particular constituents neither cleave C3 nor act as essential binding sites for passive incorporation of this complement component in epidermal basement membrane. These studies give additional support to the hypothesis that C3d,g is a previously unrecognized constituent of normal epidermal basement membrane and does not represent passive incorporation of circulating C3 at this site in human skin. *J Invest Dermatol 92:734–738, 1989*

Recent studies in our laboratory have shown that C3d,g, a 41,000-Da cleavage fragment of the third component of complement, is present in normal human epidermal basement membrane (BM) [1]. Moreover, the demonstration that C3d,g is absent from the epidermal basement membrane of a patient with congenital C3 deficiency has confirmed the specificity of this observation and dismissed the possibility that it represents the presence of a cross-reacting antigen or C3 analogue. In these studies of normal human skin, C3d,g has been found to be confined to the epidermal BM where it is found along the base of the lamina densa and within the sublamina densa region by immunoelectron microscopy. C3d,g has not been found within dermal microvascular basement membranes although recent studies have identified its presence in renal, tracheal, and placental basement membranes [1,2].

In studies of human epidermal basement membrane, papulonodular basal cell carcinomas (PNBCCs) have served as a useful model for the investigation of various BM antigens and matrix proteins. These studies have demonstrated defective expression of selected antigens in BM surrounding PNBCC tumor nests such as the absence or substantial reduction of bullous pemphigoid (BP) antigen as well as the decreased expression of anchoring fibril markers AF_1 and AF_2 [3–6]. In contrast, laminin and type IV collagen have been shown to be present in a normal amount and distribution within BMs of these tumor nests [4,7–9]. The consistency and specificity of these alterations supports the concept that these tumors are a useful substrate for the study of human epidermal BM. In this study, PNBCC have been used to extend our understanding of epidermal BM C3d,g. In addition, the distribution and intensity of C3d,g within PNBCC tumor nest BM have been compared with known antigens and matrix proteins to evaluate possible relationships between these constituents.

Manuscript received July 18, 1988; accepted for publication October 16, 1988.

Supported by NIH Grant AR37446(KBY) and NATO Scientific Affairs Subvention RGO 244/88-3/9/90 (NB-S, KBY). Dr. Basset-Séguin is a Fellow of the Ligue Nationale Francaise Contre Le Cancer.

Reprint requests to: Kim B. Yancey, M.D., Department of Dermatology, USUHS, B4080, 4301 Jones Bridge Road, Bethesda, Maryland 20814-4799.

Abbreviations:
BM: basement membrane
BP: bullous pemphigoid
EBA: epidermolysis bullosa acquisita
FITC: fluorescein isothiocyanate
PNBCC: papulonodular basal cell carcinoma

0022-202X/89/$03.50

MATERIALS AND METHODS

Tissue Samples Tissue samples from ten histologically confirmed PNBCC were provided by Dr. Parlette (Naval Hospital Bethesda, Bethesda, MD). Samples of normal adult human skin were provided by Dr. Katz (National Cancer Institute, National Institutes of Health, Bethesda, MD). Following excision, tissue samples were immediately placed in O.C.T. Compound (Lab-Tek Products, Naperville, IL), frozen, and stored at −30 to −70°C.

Reagents Rabbit anti-human C3d (Dako Corporation, Glostrup, Denmark) reacts with C3d,g as well as with its subsequent degradation product, C3d. This antibody was used to identify these C3 cleavage fragments, which are hereafter referred to as C3d,g. Previous studies [1] with specific monoclonal antibodies confirmed the presence of both C3d and C3g within normal epidermal BM and were not repeated here. Rabbit anti-type IV collagen and rabbit anti-mouse laminin were acquired from Collaborative Research, Incorporated (Bedford, MA). Sheep anti-type IV collagen was provided by Dr. Stanley (National Cancer Institute, National Institutes of Health, Bethesda, MD). KF-1, a murine monoclonal antibody that recognizes a noncollagenous component of the lamina densa of normal human epidermal BM [10], was provided by Dr. Katz. Fluorescein isothiocyanate (FITC) conjugated rabbit anti-sheep IgG (Tago Incorporated, Burlingame, CA), FITC-conjugated goat anti-mouse IgG (Tago), FITC-conjugated goat anti-human IgG (Cappel, Malvern, PA), and samples of normal rabbit, sheep, and mouse sera (Gibco, Grand Island, NY) were used as second-step and control reagents as previously described [1]. Serum samples from patients with documented BP or epidermolysis bullosa acquisita (EBA) contained IgG anti-epidermal BM antibodies at titers of 1:320 and 1:160, respectively. These sera were used as standard reagents to determine expression of these epidermal BM antigens in samples of normal human skin and PNBCC.

Immunofluorescence and Light Microscopy Studies Six-micron frozen sections of tissue were placed on albumin-coated slides and used for immunofluorescence microscopy studies. With tumor samples, every sixth tissue section was placed on an untreated glass slide, fixed in ethanol, stained with hematoxylin/eosin, and studied by light microscopy for reference purposes. Immunofluorescence microscopy was performed with the various reagents and controls as previously described [1]. All samples were tested in duplicate and independently evaluated as unknowns by at least two investigators. Immunofluorescence microscopy slides were viewed with a Leitz Ortholux II microscope equipped with a camera (Wetzler, Germany). In all PNBCC tissue sections, overlying and adjacent epidermal BM as well as BM surrounding dermal tumor nests were studied for the expression of BP, EBA, and KF-1 antigens as well as type IV collagen, laminin, and C3d,g.

RESULTS

Immunofluorescence and Light Microscopy Studies In control studies on sections of normal adult human skin, rabbit anti-human C3d bound to the epidermal BM in a continuous, somewhat stitched, linear pattern. Similar staining was observed in adnexal BM; however, as previously described, rabbit anti-human C3d did not bind dermal microvascular BM [1]. In PNBCC tumor nest BM, C3d,g was either completely absent (N = 9) or minimally detectable (N = 1) (Fig 1). Multiple tumor nest BMs within these lesions were consistently negative for C3d,g. Although one specimen did show evidence of C3d,g within some tumor nest BMs, its staining intensity was very faint. While epidermal BM directly overlying tumor nests showed reduced expression of C3d,g in four of ten BCC specimens, this C3 cleavage fragment was found in this location in all samples at sites adjacent to tumor (Fig 2 and Table I). Comparative studies with antibodies directed against other known BM antigens and matrix proteins were conducted in parallel on these tumor samples (Fig 3 and Table I). In brief, BP antigen was absent (N = 6) or notably decreased (N = 4) in BM surrounding PNBCC tumor nests. The epidermal BM overlying tumor nests was consistently positive for BP antigen, although the staining intensity of this glycoprotein was quite reduced in two samples. KF-1 antigen was absent (N = 3) or significantly decreased (N = 7) in BM surrounding PNBCC tumor nests, but present in the overlying epidermal BM of all specimens. Laminin and type IV collagen were present in a bright, linear pattern in BMs of PNBCC tumor nests, the overlying and adjacent epidermis, and the microvasculature of all samples studied. While the EBA antigen was consistently present in epidermal and tumor nest BMs of all PNBCC specimens, its staining intensity in the latter location was either somewhat (N = 3) or moderately decreased (N = 3). In PNBCC tumor nest BMs, which showed some degree of staining for C3d,g, KF-1, or BP antigen, there was significantly less staining of deep tumor lobules than of superficial ones.

Figure 1. Immunofluorescence microscopy of epidermal and PNBCC tumor nest BMs after incubation with rabbit anti-human C3d (1:20). Continuous staining is present in the epidermal BM while PNBCC tumor nests *(T)* are negative.

DISCUSSION

In this study, C3d,g has been shown to be absent or substantially diminished in BM surrounding tumor nests of PNBCC. This finding, as well as the reduction in expression of KF-1 in these lesions, represents previously undescribed defects within PNBCC tumor nest BM. In addition, these studies confirm that BP antigen is notably diminished within PNBCC tumor nest BM, while laminin, type IV collagen, and EBA antigen are present in substantial quantity. These findings demonstrate a more extensive series of defects within BM of these lesions than has previously been recognized. Specifically, alterations within each ultrastructural subregion of PNBCC tumor nest BM have been documented (i.e., BP antigen within the lamina lucida, KF-1 antigen in the lamina densa, and C3d,g along the base of the lamina densa and within the sublamina densa region). Although of varying degree, these alterations have been consistent.

The basis for these documented abnormalities within BMs of PNBCC tumor nests is not known. One possible hypothesis is that certain epidermal BM constituents are not produced by neoplastic keratinocytes within these tumors. Previous studies have demonstrated that laminin, type IV collagen, BP antigen, and EBA antigen are produced by keratinocytes [11–19]. Although the exact origin of C3d,g within epidermal BM is not known, the possibility exists

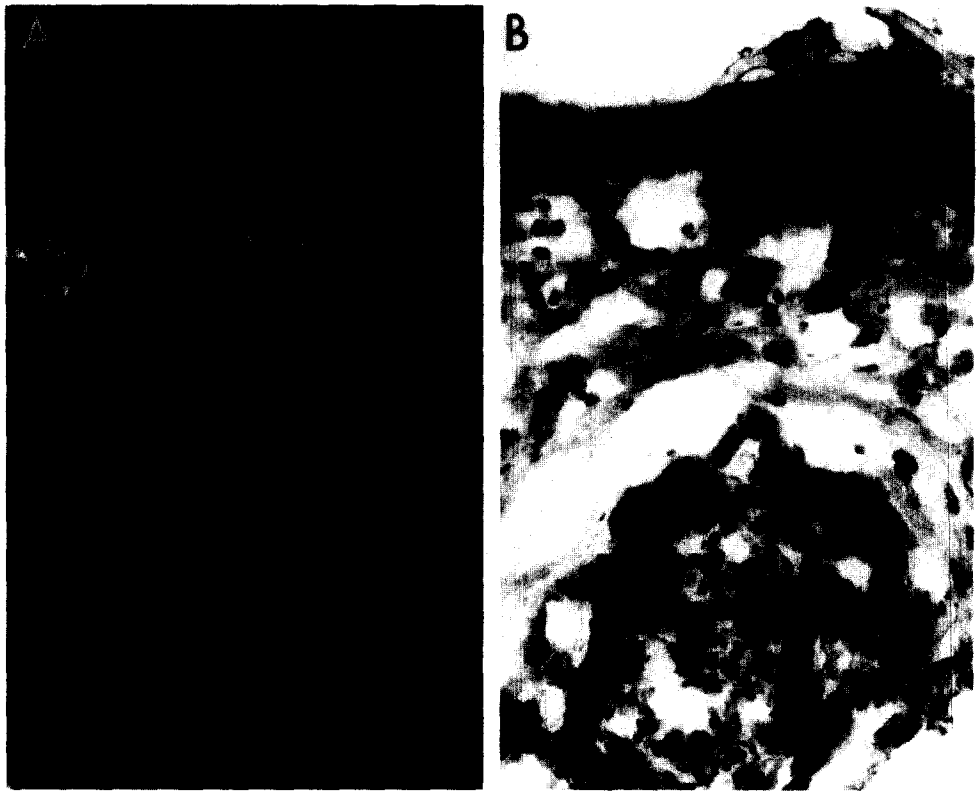

Figure 2. Immunofluorescence *(A)* and light microscopy *(B)* of sequential frozen sections of a PNBCC. C3d,g within epidermal BM overlying this PNBCC tumor nest is brightest on the *right side of the field;* the epidermal BM on the *left side of the field* exemplifies the diminished expression of C3d,g which was observed overlying tumor nests in some PNBCCs. The PNBCC tumor nest BM shows no evidence of C3d,g.

that it too may originate from keratinocytes, because recent studies have shown that A431 cells [2,20], a human cutaneous squamous carcinoma cell line, as well as normal human keratinocytes [20], synthesize and secrete C3 in vitro. If keratinocyte-derived C3 is a marker of cell differentiation (as has been postulated for BP antigen [4]), it may understandably be absent or deficient within PNBCC or overlying, possibly tumor-associated epidermal BM. Conversely, these BM constituents may be produced normally within these tumors then destroyed or altered by proteases aberrantly produced within or induced by PNBCC [21–23]. Such protease activity could also account for the reduced expression of C3d,g in epidermal BM overlying PNBCC tumor nests of selected samples in this study.

In this study, BMs of PNBCC tumor nests have also served as a useful substrate to investigate the relationship between C3d,g and other known BM antigens and matrix proteins in vivo. In this regard, C3d,g was absent from PNBCC tumor nest BMs despite the presence of laminin, type IV collagen, and EBA antigen. This lack of association suggests that the absence of C3d,g at these sites is not directly related to a corresponding deficiency of one of these BM constituents. Moreover, these constituents must not serve as essential binding sites for passive incorporation of C3 or C3d,g within epidermal BM. Interestingly, a recent study has demonstrated that C3 (as well as hydrolyzed C3) binds laminin in vitro, and that this interaction may account for the presence of C3 (in the form of C3d) within renal and placental BMs [2]. Our studies of human skin suggest an alternate hypothesis because C3d,g is localized to a different ultrastructural subregion of the epidermal BM as well as absent from laminin-rich PNBCC tumor nest and dermal microvascular BMs [1]. Moreover, the presence of laminin, type IV collagen,

Table I. Basement Membrane Antigens in Papulonodular Basal Cell Carcinomas[a]

Case No.	BP Antigen		Laminin		Type IV Collagen		KF-1 Antigen		EBA Antigen		C3d,g	
	EBM[b]	TBM[c]	EBM	TBM	EBM	TBM	EBM	TBM	EBM	TBM	EBM	TBM
1	+++	+	+++	+++	+++	+++	++	±	+++	++	+	−
2	++	−	+++	+++	+++	+++	++	±	+++	++	±	−
3	++	−	+++	+++	+++	+++	++	±	+++	+	++	−
4	++	−	+++	+++	+++	+++	++	−	+++	+	+	−
5	+	±	+++	+++	+++	+++	++	±	+++	+++	+	−
6	++	±	+++	+++	+++	+++	++	±	+++	+++	+	−
7	++	−	+++	+++	+++	+++	++	±	+++	+	+	±
8	+++	−	+++	+++	+++	+++	+++	−	+++	+++	±	−
9	+++	±	+++	+++	+++	+++	+	−	+++	++	±	−
10	+	−	+++	+++	+++	+++	+++	±	+++	+++	±	−

[a] Results of immunofluorescence microscopy are presented as follows: ±: faint; +: positive; ++: bright pattern of staining; and +++: very bright pattern of staining.
[b] EBM: epidermal basement membrane overlying PNBCC tumor nests.
[c] TBM: PNBCC tumor nest basement membranes.

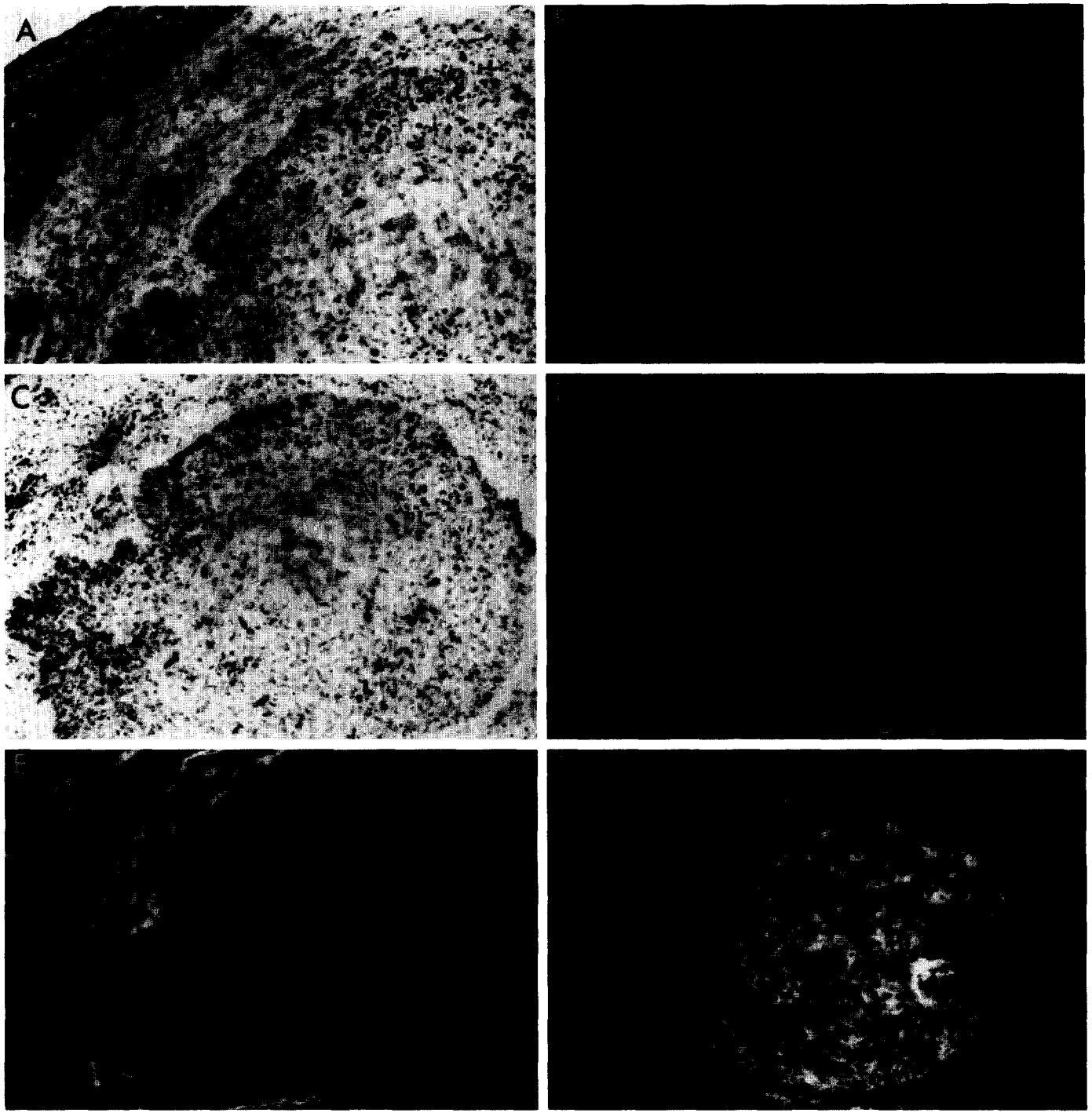

Figure 3. Light *(A,C)* and immunofluorescence *(B,D,E,* and *F)* microscopy of frozen sections of a PNBCC. Staining of a sequential section of the PNBCC shown in *panel A* with antibodies directed against laminin demonstrates the presence of this BM constituent within epidermal, microvascular, and tumor nest BMs *(B)*. Similarly, the PNBCC shown in *C* demonstrates BP antigen *(D)* and type IV collagen *(E)* within tumor nest BM which was negative for KF-1 *(F)*. As shown above, the epidermal BMs of the PNBCC shown in *panel C* was positive for each of these constituents; type IV collagen was also demonstrated in dermal microvascular BM of this specimen *(E)*.

and EBA antigen within PNBCC tumor nest BMs suggests that these matrix proteins do not produce low grade, continuous activation of circulating C3, which results in subsequent incorporation of C3d,g within epidermal BM. Although some degree of co-association exists in the defective expression of C3d,g, KF-1, and BP antigens in PNBCC tumor nest BMs, these constituents are located within different ultrastructural subregions of the epidermal BM. Hence, it is unlikely that these constituents act as essential binding or activation sites for C3 in epidermal BM.

In summary, this study has utilized PNBCC as a model substrate to extend our understanding of C3d,g within human epidermal BM and further define its relationship with other known components of this important ultrastructural region. These findings add further support to the hypothesis that C3d,g is a previously unrecognized component of normal epidermal BM and does not represent passive incorporation and local degradation of circulating C3 at this site in human skin.

REFERENCES

1. Basset-Séguin N, Dersookian M, Cehrs K, Yancey KB: C3d,g is present in normal human epidermal basement membrane. J Immunol 78:456-459, 1988

2. Leivo I, Engvall E: C3d fragment of complement interacts with laminin and binds to basement membranes of glomerulus and trophoblast. J Cell Biol 103:1091–1100, 1986
3. De Moragas JM, Winkelmann RK, Jordon RE: Immunofluorescence of epithelial skin tumors. II. Basement membrane. Cancer 25:1404–1407, 1970
4. Stanley JR, Beckwith JB, Fuller RP, Katz SI: A specific antigenic defect of the basement membrane is found in basal cell carcinoma but not in other epidermal tumors. Cancer 50:1486–1490, 1982
5. Tosca A, Varelzidis A, Nicolis G, Hadzis J, Stratigos J, Capetanakis J: Antigenic alterations in tumors of epidermal origin. Cancer 45:2284–2290, 1980
6. Lane AT, Goldsmith LA, McCoon PE, Muhlbauer JE: Decreased anchoring-fibril antigens (AF_1 and AF_2) in basal cell carcinoma. Arch Derm Res 277:499–501, 1985
7. Van Cauwenberge D, Pierard GE, Foidart JM, Lapiere CM: Immunohistochemical localization of laminin, type IV, and type V collagen in basal cell carcinoma. Br J Dermatol 108:163–170, 1983
8. Kallioinen M, Autio-Harmainen H, Dammert K, Risteli J, Risteli L: Discontinuity of the basement membrane in fibrosing basocellular carcinomas and basosquamous carcinomas of the skin: an immunohistochemical study with human laminin and type IV collagen antibodies. J Invest Dermatol 82:248–251, 1984
9. Barsky SH, Grossman DA, Bhuta S: Desmoplastic basal cell carcinomas possess unique basement membrane-degrading properties. J Invest Dermatol 88:324–329, 1987
10. Breathnach SM, Fox PA, Neises GR, Stanley JR, Katz SI: A unique epithelial basement membrane antigen defined by a monoclonal antibody (KF-1). J Invest Dermatol 80:392–395, 1983
11. Stanley JR, Hawley-Nelson P, Yaar M, Martin GR, Katz SI: Laminin and bullous pemphigoid antigen are distinct basement membrane proteins synthesized by epidermal cells. J Invest Dermatol 78:456–459, 1982
12. Timpl R, Glanville RW, Wick G, Martin GR: Immunochemical study on basement membrane (type IV) collagen. Immunology 38:109–116, 1979
13. Alitalo K, Kuismanen E, Myllyla R, Kiistala U, Askoseljavaara S, Vaheri A: Extracellular matrix proteins of human epidermal keratinocytes and feeder 3T3 cells. J Cell Biol 94:497–505, 1982
14. Petersen MJ, Woodley DT, O'Keefe EJ: Cultured human keratinocytes synthesize and secrete type IV procollagen. Clin Res 36:378A, 1988
15. Woodley D, Didierjean 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. Woodley DT, Briggaman RA, GammonWR, O'Keefe EJ: Epidermolysis bullosa acquisita antigen is synthesized by human keratinocytes cultured in serum-free medium. Biochem Biophy Res Commun 130:1267–1272, 1985
18. Stanley JR, Rubinstein N, Klaus-Kovtun V: Epidermolysis bullosa acquisita antigen is synthesized by both human keratinocytes and human dermal fibroblasts. J Invest Dermatol 85:542–545, 1985
19. Woodley DT, Briggaman RA, Gammon WR, Falk RJ, Rese MJ, Tomsick RS, O'Keefe EJ: Epidermolysis bullosa acquisita antigen, a major cutaneous basement membrane component synthesized by human dermal fibroblasts and other cutaneous tissues. J Invest Dermatol 87:227–231, 1986
20. Basset-Séguin N, Yancey KB: A431 cells and normal human keratinocytes synthesize and secrete the third component of complement. Clin Res 36:632A, 1988
21. Liotta LA, Tryggvason K, Garbisa S, Hart I, Foltz CM, Shafie S: Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 284:67–68, 1980
22. Goslen JB, Eisen AZ, Bauer EA: Stimulation of skin fibroblast collagenase production by a cytokine derived from basal cell carcinomas. J Invest Dermatol 85:161–164, 1985
23. Barsky SH, Siegal GP, Jannotta F, Liotta LA: Loss of basement membrane components by invasive tumors but not by their benign counterparts. Lab Invest 49:140–147, 1983