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Origin of Basement Membrane Type IV Collagen in Xenografted Human Epithelial Tumor Cell Lines

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The origin of basement membrane (BM), deposited in epithelial neoplasms, was studied in xenografts of human tumor cell lines in nude mice and rats. Cell lines were chosen that* in vitro *do (WISH, KB) or do not (5583-E; HT-29) produce BM components, more specifically, type IV collagen. Basement membrane deposition was studied by immunohistochemistry, using species cross-reactive polyclonal anti-human type IV collagen antiserum, mouse- and rat-specific polyclonal anti-mouse type IV collagen antiserum, human-specific monoclonal anti-type-IV collagen antibody, and by* in situ *hybridization, using a ^{32}S-labeled species cross-reactive cDNA probe, specific for type IV collagen mRNA. In the xenografts, species-cross-reactive anti-type-IV collagen antiserum demonstrated the presence of irregular and discontinuous BM. In 5583-E and HT-29 xenografts, only murine type IV collagen epitopes were detected. In contrast, in WISH and in KB xenografts, the BM stained human as well as murine type IV collagen epitopes. By* in situ *hybridization, type IV collagen mRNA was detectable in stromal cells only in 5583-E and HT-29 xenografts, but in both epithelial and stromal cells in WISH and KB xenografts. These results indicate that in this model system epithelial tumor cells and stromal (mesenchymal) cells are involved in the production and deposition of a BM. (Am J Pathol 1990, 136:1165–1172)

Basement membranes (BM), which occur at the interface between epithelia and the underlying stroma, are composed of macromolecules such as type IV collagen,[1] laminin,[2,3] and heparan sulphate proteoglycan.[4] Using specific antibodies to one or more of these components, it has been shown that, in many carcinomas, a discontinuous BM is deposited at the epithelial-stromal interface. It has been suggested that the ability of cancer cells to deposit or degrade a BM is associated with invasive and metastatic behavior[5–10] and is correlated with patient survival.[11]

It is generally assumed that epithelial BM are at least partly a product of the epithelial cells.[12,13] Several investigators have demonstrated that *in vitro* epithelial tumor cells may continue to produce BM components.[14–22] However, evidence has been presented that mesenchymal cells also are able to produce BM components and may therefore contribute to the formation of the BM. Kühl et al[23] and Sanderson et al[24] showed, by cocultivation of mouse muscle fibroblasts and quail skeletal myoblasts, that fibroblasts deposit the myoblast BM. Similarly, cultivation of chick mesenchyme and rat endoderm resulted in formation of a BM of mesenchymal origin.[25]

Experimental data concerning BM origin in neoplasms *in vivo* is very scanty. Recently, Damjanov et al[26] studied the extracellular matrix of human tumor xenografts in nude mice using monoclonal anti-laminin antibodies, exclusively reactive with human laminin, and polyclonal anti-laminin antisera reacting with human as well as with murine laminin. In many of their xenografts, tumoral BM laminin appeared to be both of human and murine origin. They concluded that laminin in the extracellular matrix of xenografted tumors is at least partly of stromal origin. In the present report, we have extended these investigations with regard to type IV collagen production in xenografts of human tumor cell lines in nude rats. Two human cell lines were used that do and two that do not produce type IV collagen *in vitro*. The origin of type IV collagen in the rat xenografts was investigated using a type IV collagen cDNA probe for detection of type IV collagen mRNA by *in situ* hybridization and species-specific antibodies to type IV collagen for immunohistochemistry.

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Material and Methods

Tissues

Human cell lines derived from colonic adenocarcinoma (5583-E, HT-29), from transformed amniotic epithelium (WISH) and from an oral epidermoid carcinoma (KB), were used in this study. The characteristics of the 5583-E and HT-29 cell lines are described extensively elsewhere.[27,28] The WISH and KB cell lines were obtained from the American Type Culture Collection (ATCC). The characteristics of these cell lines were reported by Hayflick and Eagle.[29,30] Cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM, Flow Laboratories, Amstelstad, Zwanenburg, The Netherlands) supplemented with 10% fetal calf serum (FCS, Boehringer, Mannheim, West Germany), before xenografting.

Human type I collagen was isolated from placenta as described elsewhere.[31] Cells were cultured in or on human type I collagen lattices (1 or 2 mg collagen per milliliter), as described by Nusgens.[32] Lattices were sometimes populated with human skin fibroblasts, which contract the lattice within a few days. For histologic evaluation, the collagen lattices were processed as described for the tissue samples.

Xenografting

All tumor cell lines were xenografted in nude mice (NMRI nu/nu supplied by Zentral Institut für Versuchstiere, Hannover, West Germany) and nude rats (CD1 nu/nu supplied by Charles River, Sulzfeld, West Germany) by injecting tumor cell suspensions (1.5×10^6 cells in 100 to 200 μl phosphate-buffered saline [PBS]) subcutaneously. After a period of 2 to 6 weeks, solid tumors of 0.5 to 1 cm in diameter were obtained.

Xenografts were removed and samples of the xenografts and the collagen lattice cultures were snap-frozen in isopentane quenched in liquid nitrogen and stored at −70°C and/or fixed in 4% buffered formaldehyde and further embedded in paraffin.

Antibodies

Polyclonal cross-species–reactive antibodies were generated by immunization of rabbits with human type IV collagen isolated by pepsin digestion from human placenta, as extensively described elsewhere.[31] Monoclonal antibodies (1042 and 1043) were obtained after immunization with pepsin cleaved type IV collagen from human placenta.[31] Monoclonal antibody 1043 is human-specific, whereas 1042 reacts with human and rabbit type IV collagen.[31] A polyclonal rabbit anti-mouse type IV collagen was provided by Drs. M. Demarchez and D. J. Hartmann.[33] This antibody was absorbed repeatedly with human type IV collagen, and was used in a 1:1000 to 1:2000 dilution.

Mouse monoclonal anti-human laminin (4E10)[34] is human-specific and reacts with all BM. A rabbit polyclonal anti-mouse laminin was obtained by immunizing rabbits with mouse Engelbreth-Holn-Swarm (EHS) tumor laminin (E/Y Laboratories, San Maleo, CA). This polyclonal antibody is reactive with all BM, is cross-species–specific, and does not react with type IV collagen or fibronectin on dot blots and western blots.

Immunoblotting of Culture Supernatants

Tissue culture supernatants of 5583-E, HT-29, WISH, and KB cells were precipitated with 4 mol/l (molar) NaCl, dissolved in and dialyzed against sodium dodecyl sulfate (SDS)-sample buffer and run on a 5% SDS-polyacrylamide gel,[35] followed by blotting onto nitrocellulose membrane.[36] The nitrocellulose filters were washed (three times 10 minutes) with PBS and 0.05% Tween 20 (Serua, Heidelberg, FRG), and subsequently incubated (1 hour at room temperature) with the specific antisera, diluted in PBS and 0.1% bovine serum albumin (BSA, St. Louis, MO). The blots were washed (three times 10 minutes) with PBS and 0.05% Tween 20 and incubated with the peroxidase-labeled conjugate, either goat anti-rabbit gamma G immunoglobulin (IgG) or rabbit anti-mouse IgG (Dakopatts). Finally, the blots were washed three times for 10 minutes and developed using 0.5 mg/ml 3,3′ diaminobenzidine diluted in 50 mmol/l (millimolar) TRIS pH 7.6 and 0.002% H_2O_2, washed again, and air dried.

Immunohistochemistry

In order to expose the antigenic sites, for all antigens, paraffin tissue sections were pretreated with pronase (Boehringer, 400 μg/ml in 50 mmol/l TRIS-HCl buffer, pH 8.0, for 30 minutes at 37°C) when monoclonal antibodies were applied, or with pepsin (Boehringer, mg/ml in 0.01 N HCl, 30 minutes at room temperature) for the polyclonal antisera. On frozen sections, the antibodies could be employed without enzyme pretreatment. Sections were incubated for 1 hour with the primary antibodies and subsequently with peroxidase-labeled rabbit anti-mouse IgG or peroxidase-labeled goat anti-rabbit IgG (Dakopatts, Copenhagen, Denmark) for the monoclonal and polyclonal antibodies, respectively. After each incubation, sections were washed with PBS (three times for 5 minutes). Immunoreactive sites were visualized with diaminobenzidine. Finally, the sections were lightly counterstained with hematoxylin.

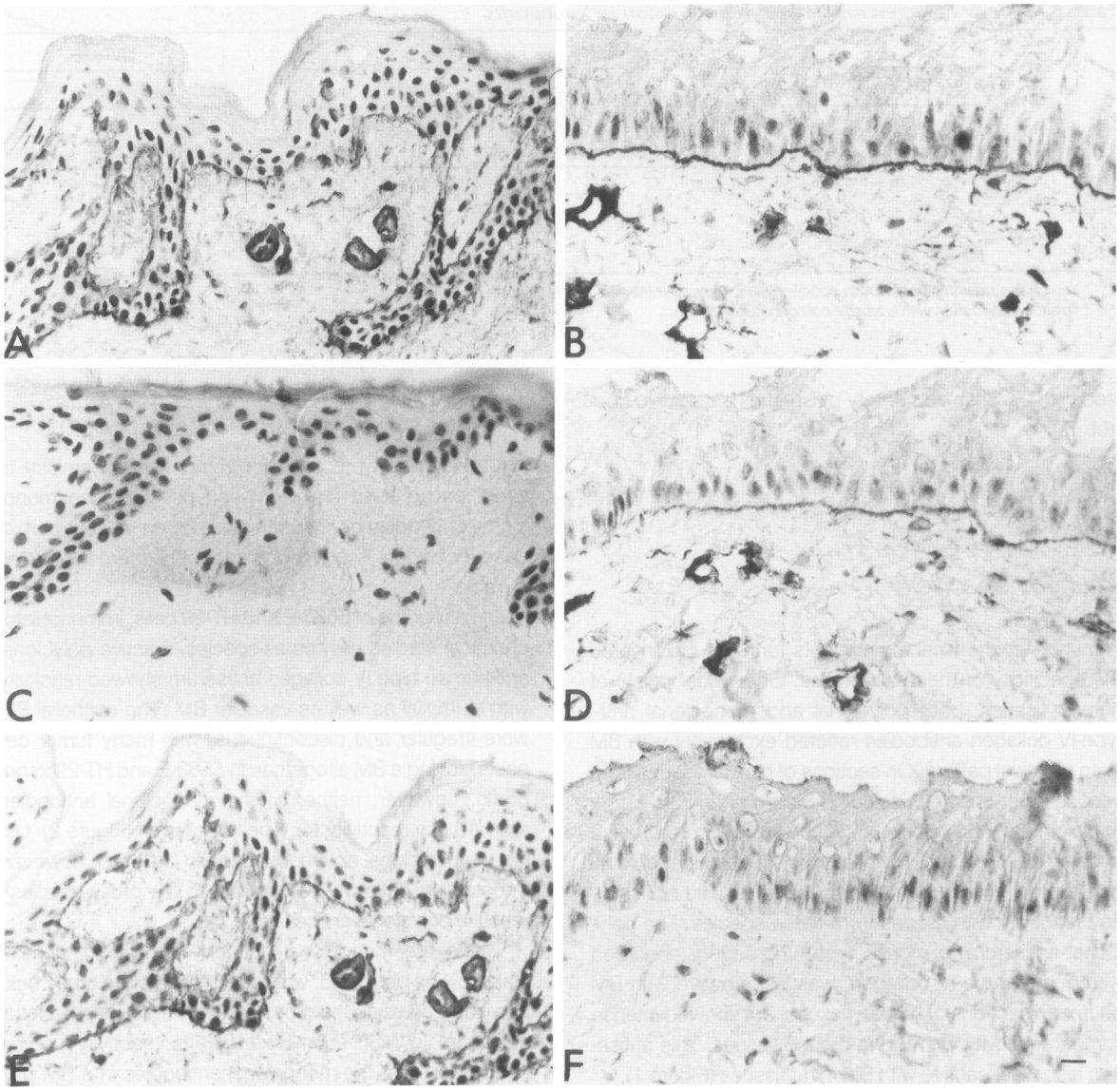

Figure 1. *Immunoreactivity on frozen sections from human skin* **(A, C, E)** *and rat skin* **(B, D, F)** *with polyclonal anti-human type IV collagen antiserum* **(A, B)**, *polyclonal anti-mouse type IV collagen antiserum* **(C, D)**, *monoclonal (1042) anti-human type IV collagen antibody* **(E, F)**. *Scale bar = 10 μ.*

In Situ *Hybridization*

Frozen sections (4 μ) were mounted on RNAse-free coated slides, fixed with freshly prepared 4% paraformaldehyde, and washed with PBS, baked, washed again, and treated with Triton X-100. After washing in PBS, the sections were incubated with proteinase K in a buffer containing TRIS and ethylenediaminetetraacetic acid (EDTA).[37] The sections were fixed again with 4% buffered paraformaldehyde, washed with PBS, and treated with acetic anhydride in triethanolamine, and furthermore incubated with formamide in 2× sodium saline citrate (SSC). ^{35}S-dCTP–labeled type IV collagen probe[38] was prepared according to the random primed labeling method using a Boehringer labeling kit.[39,40] A probe/hybridization mixture (15 μl), containing 300,000 cpm, was applied to each section. The sections subsequently were covered with 22-×22-mm siliconized coverslips and sealed with rubber cement. Hybridization mixture contained yeast tRNA, salmon sperm DNA, deionized formamide, TRIS-HCl, NaCl, EDTA, Denhardt's mixture, Dextran sulphate, and dithiothreitol (DTT). After 12 to 16 hours' incubation at 50°C, the coverslips were removed and the sections were washed with SSC. After these steps, the sections were dehydrated in graded series of ethanol, air-dried, and coated with Kodak NTB-2 nuclear track emulsion and exposed for various periods. The exposed slides were developed in 1:1 Kodak (Eastman Kodak, Rochester, NY) D-19 developer, fixed, and counterstained with hematoxylin, dehydrated, and mounted. Parallel sections were hybrid-

Table 1. *Type IV Collagen and Laminin Production in Xenografts*

	Type IV collagen					Laminin	
	IHC*			ISH†		IHC*	
Cell line	Cross-reactive	Human	Mouse/rat	Tumor cells	Interstitium	Cross-reactive	Human
5583	+	−	+	−	+	+	−
HT-29	+	−	+	−	+	+	−
KB	+	+	+	+	+	+	+
WISH	+	+	+	+	+	+	+

* Immunohistochemistry; antibody specificity as outlined in Materials and Methods.
† *In situ* hybridization with a type IV collagen cDNA probe.

ized with a ^{35}S-labeled empty plasmid as a negative control.

Results

Antibody Specificity

Immunohistochemical experiments further substantiated the specificity of these antibodies. On a wide variety of human tissues, both polyclonal and monoclonal anti-type-IV collagen antibodies reacted exclusively with BM in an identical pattern. On sections of normal tissues from various other species (including Balb/c and NMRI nu/nu mice or rats), polyclonal anti-type-IV collagen antisera showed a similar pattern of reactivity. The two monoclonal anti-type-IV collagen antibodies, however, did not show any reactivity with tissues from these species. The polyclonal antiserum to mouse type IV collagen, absorbed with human type IV collagen, was not reactive with BM on frozen sections of human tissues, but showed intense immunoreactivity with BM in mouse tissues. This antiserum also was reactive with BM in rat tissues (Figure 1).

Type IV Collagen Detection in Cancer Cell Lines

Immunohistochemical studies of 5583-E or HT-29 cells grown in artificial collagen lattices did not reveal reactivity with any of the polyclonal or monoclonal anti-type IV collagen antibodies. On immunoblots of precipitated tissue culture supernatants of 5583-E and HT-29, immunoreactivity with antibodies to type IV collagen did not occur. In contrast, pro α1- and pro α2-chains of type IV collagen were detected in supernatants of WISH and KB cell lines.

Cultured in collagen lattices, WISH and KB cells showed extensive granular intracytoplasmic and/or pericellular immunoreactivity for type IV collagen with polyclonal as well as monoclonal anti-type-IV collagen antibodies. However, BM-like structures could not be identified.[41]

Type IV Collagen Detection in Xenografts

Rat xenografts were used for immunohistochemistry to avoid background staining when using mouse monoclonal antibodies on mouse tissues in an indirect peroxidase technique. The immunoreactivity patterns are listed in Table 1.

In xenografts of 5583-E and HT-29 cells, immunohistochemical studies with cross-species–reactive polyclonal anti-human type IV collagen antiserum showed reactivity with epithelial as well as vascular BM. The epithelial BM were irregular and discontinuous, with many tumor cell nests lacking a BM altogether. In 5583-E and HT-29 xenografts, however, neither of the monoclonal antibodies showed any reactivity for type IV collagen (Figure 2). Using the polyclonal anti-mouse type IV collagen, however, the staining pattern was identical to the cross-species–reactive polyclonal antiserum.

Using the monoclonal antibody to laminin, no BM staining was observed, whereas BM-like structures could be visualized using the cross-species–reactive polyclonal antiserum. In WISH and KB xenografts, reactivity for type IV collagen with the monoclonal antibodies in a BM pattern could be demonstrated clearly (Figure 2).

Not only human-specific type IV collagen, but also human laminin could be demonstrated in these xenografts. Basement membranes of vascular structures in the xenografts did not react with the monoclonal anti-type-IV collagen antibodies.

The polyclonal antiserum specific for mouse/rat type IV collagen and the cross-species–reactive anti-human type IV collagen antiserum showed identical staining in all the xenografts. These antibodies also stained the BM of blood vessels, Schwann cells, muscle cells, and fat cells.

In Situ *Hybridization*

The labelling patterns in *in situ* hybridization experiments, using a type IV collagen cDNA probe, are listed in Table 1. On frozen sections of the xenotransplants of 5583-E and HT-29, we observed intense labeling of the host stro-

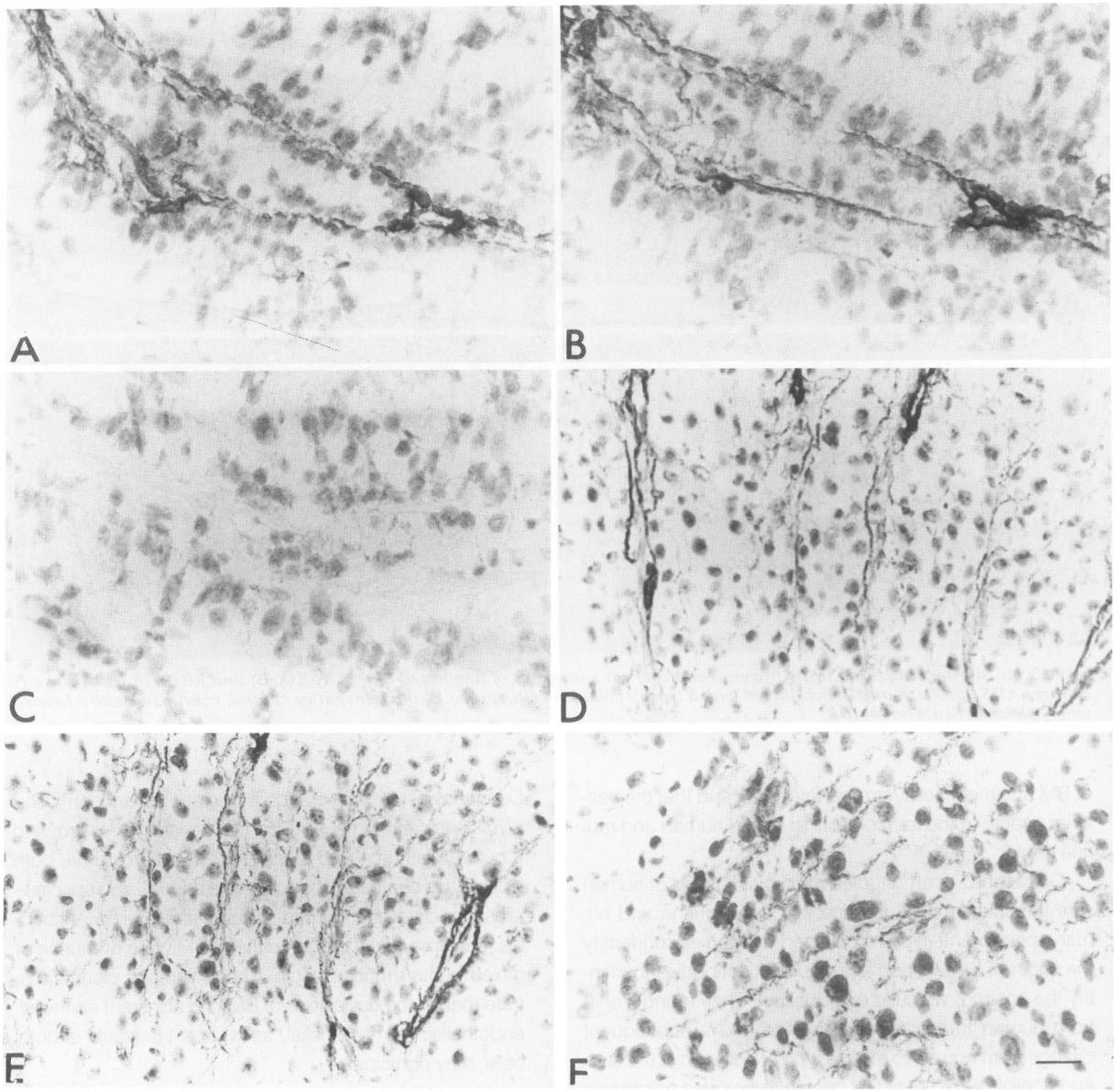

Figure 2. *Frozen sections of rat xenografts of tumor cell-line 5583-E* **(A, B, C)** *and KB* **(D, E, F)** *(immunoperoxidase).* **A** *and* **D**: *cross-species-reactive polyclonal antiserum to type IV collagen.* **B** *and* **E**: *mouse- and rat-specific polyclonal antiserum to type IV collagen.* **C** *and* **F**: *human-specific monoclonal antibody to type IV collagen (MA 1042). Scale bar = 10 μ.*

mal cells adjacent to the human tumor cells, but not of the tumor cells (Figure 3).

Experiments on frozen sections of KB and WISH xenotransplants showed a slight but distinct hybridization labeling of tumor cells and also of stromal cells (Figure 3). The used cDNA probe also recognized rat type IV collagen mRNA, because in all xenografts labeling was noticed of basal cells of the rat epidermis and also of some mesenchymal cells of the dermis adjacent to the epidermal BM or surrounding the xenografted tumor cells (Figure 4).

Discussion

In xenografts of human tumor cell lines in nude mice or rats, the interaction *in vivo* between neoplastic epithelial cells and mesenchymal stromal elements can be investigated. For example, the origin of the BM can be traced when antibodies that discriminate between human and murine BM components are employed in immunohistochemical studies. Using this approach, Holmstrup[42] studied the immunophenotype of laminin and type IV collagen in BM of normal human oral mucosa transplanted into nude mice. He found that species-cross-reactive polyclonal antisera against laminin and type IV collagen reacted equally well with vascular and epithelial BM, whereas human-specific monoclonal antibodies reacted intensely with vascular BM, but only weakly or not at all with epithelial BM. Holmstrup concluded that, under these conditions, oral mucosal cells participate in the deposition

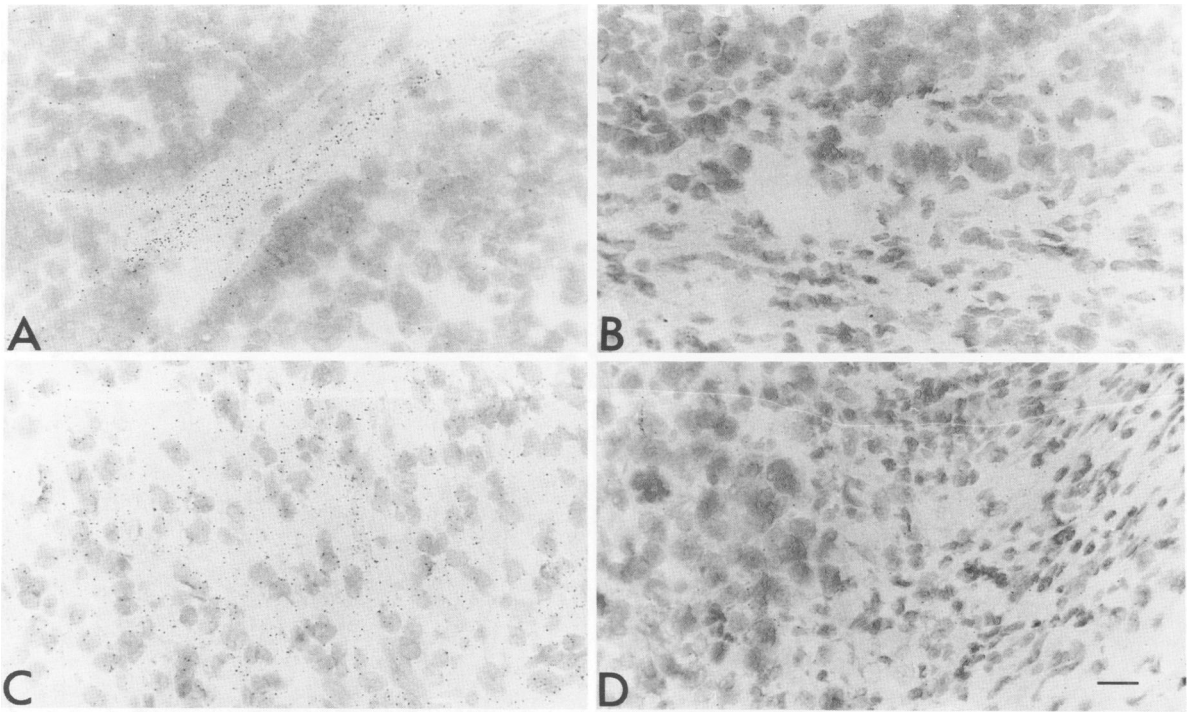

Figure 3. In situ *hybridization using frozen sections of rat xenografts of tumor cell-line 5583-E* **(A, B)** *and KB* **(C, D)** *with a type IV collagen cDNA probe.* **A** *and* **C**: *hybridized with a type IV collagen cDNA probe.* **B** *and* **D**: *negative control, hybridized with a labeled empty plasmid. Scale bar = 10 μ.*

of BM at the epithelial/stromal interface, and that the vascular supply of the transplant is of mixed human and murine origin.

Demarchez et al[33] studied revascularization of human skin transplants in nude mice, applying murine and human-specific anti-type-IV collagen antibodies to identify the origin of the BM and anti-HLA-DR antibodies to identify the origin of vascular endothelium. They found a shift from human to murine endothelium and a subsequent change from human to mouse type IV collagen in the subendothelial BM. Kühl et al[23] cocultured mouse myoblasts with chicken fibroblasts and found deposition of chicken type IV collagen in the BM around mouse myoblasts.

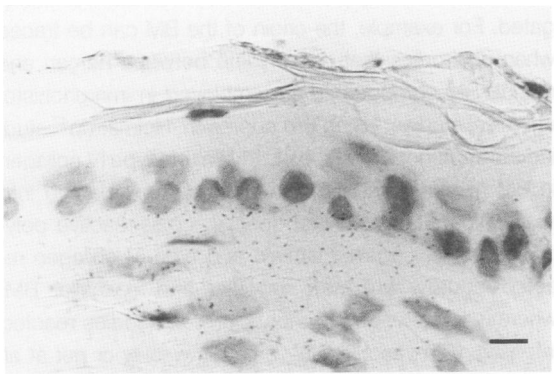

Figure 4. In situ *hybridization using frozen sections of rat epidermis with a type IV collagen cDNA probe. Scale bar = 10 μ.*

Damjanov et al[26] xenografted a lung adenocarcinoma and a yolk sac carcinoma, both producing laminin *in vitro*, and a hepatoblastoma and a hepatocellular carcinoma, both lacking this characteristic, into nude mice. Using species-cross-reactive and human-specific anti-laminin antibodies, it was shown that the epithelial BM were of murine or mixed human/murine origin. These findings clearly illustrate that, in the deposition of BM, the adherent epithelial, endothelial, or mesothelial, as well as interstitial stromal cells, may participate.

Against this background, we studied BM in human tumor cell lines, xenografted into nude mice and rats, using species-cross-reactive as well as human-specific and mouse- or rat-specific anti-type IV collagen antibodies. We used two cell lines that do and two cell lines that do not produce laminin and type IV collagen *in vitro*. The human colon cancer cell lines 5583-E[27] and HT-29[28] in immuno(histo)chemical experiments did not produce type IV collagen, nor could type IV collagen mRNA be detected by *in situ* hybridization. Nevertheless, in xenografts of these cells in nude mice and rats, fragments of BM were detected by immunohistochemistry, using a polyclonal species-cross-reactive anti-type-IV collagen antiserum. Using mouse- and rat-specific anti-type-IV collagen antibodies, we could demonstrate the type IV collagen to contain mouse- or rat-specific but not human-specific epitopes. Furthermore, we demonstrated type IV collagen mRNA in the stromal cells but not in the (xenografted)

carcinoma cells. These findings prove that type IV collagen in the epithelial BM in xenografts of 5583-E and HT-29 cells is deposited by stromal cells.

Both KB and WISH cells produced type IV collagen *in vitro*.[41] By immunohistochemistry, intracytoplasmic type IV collagen immunoreactivity was detectable, and by immunoblotting, this BM component was identified in the tissue culture supernatant. Furthermore, by *in situ* hybridization, we detected type IV collagen mRNA in these cells. When xenografted into nude mice and rats, these cell lines gave rise to tumors with extensive BM deposition. In these BM, human as well as murine type IV collagen epitopes were detected by immunohistochemistry. Furthermore, *in situ* hybridization demonstrated type IV collagen mRNA in the epithelial and in the stromal cells. In these xenografts, therefore, the epithelial BM contained components that were derived from both epithelial and stromal cells. *In situ* hybridization experiments on rat skin using the type IV collagen cDNA probe showed that type IV collagen mRNA is present in the basal cells of the epidermis, as well as in the underlying dermal cells. This observation suggests that normal epithelial BM may be also contain components of epithelial and stromal origin.

Which stromal cells could be responsible for the production of the epithelial BM components? Additional experiments in neoplasms and in normal tissues are necessary to resolve this question. It is tempting to speculate, however, that myofibroblasts are involved. Myofibroblasts play an important role in tissue repair and the desmoplastic tissue response,[43,44] and are capable of producing a variety of BM components. Furthermore, in neoplasms, often a desmoplastic reaction can be observed that consists largely of myofibroblasts, which deposit large amounts of BM material in the extracellular matrix.

In conclusion, our results demonstrate that in immunedeficient mice or rats, the BM surrounding xenografted human cancer cells is (partly) derived from murine/rat and therefore stromal cells and is partly tumor-cell–derived. It is suggested that in this situation, but probably also in normal BM, stromal myofibroblasts participate in the deposition of the BM at the interface of epithelium and mesenchymal stroma.

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