Epithelial-stromal interactions in colon cancer

FRED T. BOSMAN[1]*, ADRIAAN DE BRUÏNE[2], CLAUDIE FLOHIL[1], ANNEKE VAN DER WURFF[2], JOOP TEN KATE[2] and WINAND W.M. DINJENS[1]

[1]*Department of Pathology, Erasmus University of Rotterdam, Rotterdam, and* [2]*Department of Pathology, University of Limburg, Maastricht, The Netherlands*

ABSTRACT In this paper investigations concerning the interactions at the interface between tumor cells and tumor stroma are reviewed. As a model for tumor cell-extracellular matrix interaction human colorectal carcinoma cell lines, *in vitro* and *in vivo*, in nude mouse xenografts, were chosen. Based on the available data and on a review of the literature the following conclusions can be drawn. Most malignant epithelial neoplasms at the site of tumor cell invasion display defects in the epithelial basement membrane. This is not merely the result of enzymatic dissolution but rather reflects a shift in basement membrane turnover towards degradation. Elsewhere in the same primary tumor or in a metastasis the balance might be shifted more towards basement membrane deposition. The tendency of a tumor to deposit basement membranes reflects the biological potential of the neoplasm. Basement membranes are deposited by stromal cells or by a concerted action of tumor and stromal cells. Differentiation in a carcinoma is modulated by factors in the extracellular matrix. Endocrine differentiation can be induced *in vitro* by native basement membranes but also by direct contact of the tumor cells with fibroblasts. Basic FGF is one of the extracellular matrix factors with differentiation inducing capacity. Expression of cell adhesion molecules and integrin receptors tends to be down-regulated in carcinoma cells. Alterations in the expression of these proteins might not be constitutive but rather modulated by the direct environment of the tumor cell and might not only include quantitative alterations but also changes in their cell surface distribution, causing or following loss of cell polarity.

KEY WORDS: *colon cancer, extracellular matrix, basement membrane, integrins, E-cadherin*

Introduction

In cancer research the cancer cell itself is most frequently the object of interest. It has become clear, however, that a cancer not only consists of neoplastic cells but also contains a stromal infrastructure, including tumor vasculature, which is provided by the host. In fact, a neoplasm constitutes a unique microenvironment in which various subpopulations of tumor cells and tumor stroma interact and together determine the behavior of the neoplasm. A fascinating aspect of the stromal compartment of a tumor is that it appears to be not only a passive scaffold or an inert supply system for cellular nutrients but an active regulatory element. It is in a way responsible for the existence of the tumor: without host stroma there would be no cancer. Stromal factors modulate tumor cell growth and tumor cell differentiation, both important determinants of tumor behavior. Extracellular matrix elements, especially the epithelial basement membrane, on the one hand constitute a barrier against invasive tumor cell growth, but on the other hand they also guide tumor cells in their migration during the process of invasion.

In this paper we will focus on tumor cell-extracellular matrix (ECM) interaction. We will especially highlight the role of the basement membrane in tumor cell invasion and in tumor cell differentiation. Attention will also be paid to the origin of basement membranes in neoplasms and to the expression of cell adhesion molecules in cancer cells, in the way they function in the processes of intercellular and cell-matrix adhesion. The studies we will discuss largely concern human colon cancer cell models *in vitro* and *in vivo* but the concepts can be extrapolated to other tumor cell systems.

Subcellular and molecular architecture of the epithelial stromal interface

In a normal colon mucosa the epithelial cells are interconnected by specific cellular junctions, structurally recognizable domains of

Abbreviations used in this paper: ECM, extracellular matrix; bFGF, basic fibroblast growth factor; TGFα, transforming growth factor α; TGFß, transforming growth factor ß; CAM, cell adhesion molecule; RGD, arginine-glycine-aspartic acid.

***Address for reprints**: Department of Pathology, Erasmus University of Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. FAX: 3110.4088152.

0214-6282/93/$03.00

the plasma membrane, and by molecular adhesives. The latter, to which this discussion will be limited, comprise the cell adhesion molecules (CAM) and the integrin receptor family.

Cell adhesion molecules (CAM) fall into two categories: those that require Ca^{++} (the cadherins) and those that do not require Ca^{++}. Ca^{++} independent CAMs can be subdivided into cell-CAMs, which form homophilic interactions between epithelial cells, and a family of N(neural)-CAMs, which form homophilic interactions and also interactions with ECM proteins such as heparansulphate proteoglycan. The N-CAMs are derived from a single gene by alternative splicing and play a role in nerve and muscle development. The cadherins, of which three distinct types have been characterized (E- , P- and N-) are transmembrane glycoproteins with a MW between 120 and 135 kD. The cadherins form homophilic interactions and also play a key role in embryonal development. E-cadherin in particular, which is also known as L-CAM or uvomorulin, has generated considerable interest. It is primarily located between epithelial cells at the site of belt desmosomes, where it anchors actin filaments to the plasma membrane via actin-binding proteins. Inhibition of E-cadherin expression or blocking of its function by monoclonal antibodies leads to disruption of intercellular adhesion of epithelial cells. Without E-cadherin, none of the intercellular junctions are formed (Takeichi, 1991).

Integrins are important actors in the interaction between epithelial cells and the extracellular matrix. The family of integrin receptors comprises heterodimeric molecules, containing an α and a ß chain. So far, at least 8 α chains and 4 ß chains have been identified but this field is rapidly developing and it is not unlikely that new integrins will be discovered. For epithelial cell-stromal interactions the group of $ß_1$ integrins is especially important. The $ß_1$ integrins are also known as very late activation (VLA) antigens. This group shares a common $ß_1$ chain but the members differ in the composition of the complementary α chain (Hemler, 1990). Molecular and functional characteristics of the $ß_1$ integrins are summarized in Table 1.

Integrin receptors not only mediate cell-extracellular matrix interaction, but also may act as intercellular adhesives. For epithelial cells it is likely that $\alpha_2ß_1$ and $\alpha_3ß_1$ (VLA-2 and VLA-3) form homophilic intercellular interactions in addition to their function as laminin (VLA-3) or collagen (VLA-2, VLA-3) receptors. In colon mucosa these antigens therefore are found on the basolateral surface of epithelial cells. The $\alpha_6ß_1$ integrin (VLA-6) is a laminin receptor and therefore occurs predominantly on the basal plane of the cell, where it faces the basement membrane.

TABLE 1

THE $ß_1$ INTEGRIN FAMILY OF EXTRACELLULAR MATRIX RECEPTORS

integrin	alternative name	ligand
$\alpha_1ß_1$	VLA-1	lam, coll
$\alpha_2ß_1$	VLA-2, ECMR II	coll, c-c
$\alpha_3ß_1$	VLA-3, ECMR I	lam, coll, fin, c-c
$\alpha_4ß_1$	VLA-4, LPAM-1	fin, VCAM-1
$\alpha_5ß_1$	VLA-5, FNR, ECMR VI	fin
$\alpha_6ß_1$	VLA-6	lam

VLA= very late activation antigen; ECMR= extracellular matrix receptor; lam= laminin; coll = collagen; c-c= cell-cell interaction; fin= fibronectin; FNR= fibronectin receptor; VCAM= vascular cell adhesion molecule; LPAM= lymphocyte Peyers patch adhesion molecule.

At the interface between the plasma membrane and the ECM the epithelial cells are bordered by the basement membrane. This is a 60-100 nm-wide sheet-like structure which has been shown to consist of a network of type IV collagen fibrils, to which the other constituents of the basement membrane, including laminin, entactin/nidogen and heparansulphate proteoglycan are attached (Martinez-Hernandez and Amenta, 1983). Laminin adheres to specific laminin receptors, which partly (Mafune *et al.*, 1990) belong to the $ß_1$ integrin family on the cell surface. In this interaction an arg-gly-asp (RGD) sequence or closely related sequences play an important role. Not all laminin receptor binding involves RGD sequences, however. In the large (900 kD) cross-shaped molecule of laminin multiple domains have been recognized, with different functions. One of these is adhesion to the globular domain of type IV collagen through a type IV collagen binding site in the center of the cross (Sasaki *et al.*, 1988).

In view of its borderline position it is not unexpected that the basement membrane displays structurally and functionally different domains. Towards the cell surface the basement membrane is often less dense and a higher concentration of laminin seems to occur there. Towards the interstitial connective tissue also specific features can be recognized which are related to the adhesion between basement membrane and interstitial collagens. This is most clearly demonstrated in the lamina fibroreticularis, consisting of anchoring fibrils, which anchor type IV collagen to interstitial collagen types I and III. The lamina fibroreticularis is well developed in stratified squamous epithelia but also around glandular duct epithelia. The anchoring fibrils consist of type VII collagen (Lunstrum *et al.*, 1987). In other epithelia, however, the adhesion between the basement membrane and interstitial collagens must be mediated by other ECM components because there type VII collagen is absent. This area has not been fully explored as yet.

Functional aspects of epithelial stromal interaction

The basement membrane and the connective tissue stroma have long been regarded as passive tissue components, acting only as a scaffold for the functionally important epithelial cells. Recent studies have shown, however, that the basement membrane is a dynamic structure with a constant turnover, that stromal factors play an important role in the regulation of cellular growth and differentiation and that the development of a stromal tissue compartment is modulated by factors secreted by the adjacent epithelial cells.

Basement membrane dynamics

In normal mature tissues the basement membrane is quite stable. The turnover rate of its components has not been well established but in a normal basement membrane this is rather slow (Reddi, 1985). In the degradation of the basement membrane a variety of proteolytic enzymes plays a role. These include non-specific proteases (e.g. trypsin, cathepsins), the plasmin system (through activation by tissue-type plasminogen activator) which probably acts mostly as activator of inactive precursor enzymes, but also proteases specific for type IV collagen (Tryggvason, 1989). The latter have been extensively investigated in connection with tumor invasion and will be reviewed later. In developing tissues as well as in tissue repair and in neoplasia basement membrane turnover is dramatically increased.

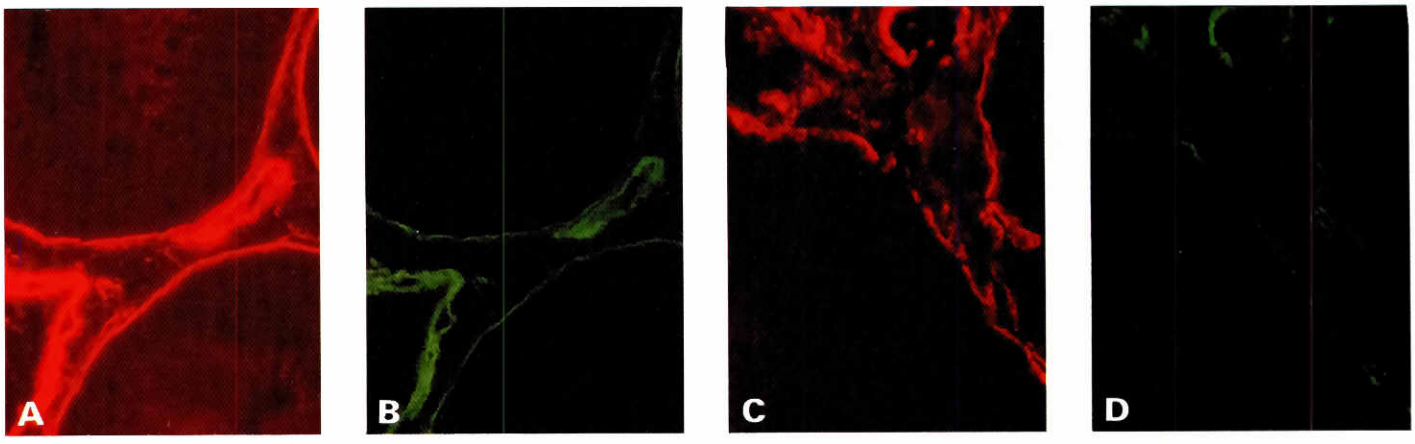

Fig. 1. Immunofluorescence detection of basement membrane type IV collagen (A, C) and laminin (B, D) in tubular adenoma (A, B) and adenocarcinoma (C, D) of the colon. *Note co-distribution of type IV collagen (rhodamine labeling) and laminin (fluorescein labeling) and the discontinuity of the basement membrane in adenocarcinoma (double indirect immunofluorescence; x250).*

The basement membrane has long been considered largely a product of the adjacent cell. Recent data has shown, however, that it is at least a product of both epithelial and stromal cells (Simon-Assman *et al.*, 1988). Situations are conceivable, and have been described, in which basement membranes are exclusively the product of stromal cells.

Stromal regulation of epithelial growth and differentiation

In a variety of systems it has been demonstrated that the function of epithelial cells is modulated by stromal factors. This is particularly clear in tissue development during organogenesis as exemplified in *in vitro* experiments in the developing prostate by Cunha (1976) and *in vivo* in classical chicken-quail xenografting experiments with regard to the induction of endocrine differentiation in the gastrointestinal tract by Andrews (1985). These and other studies have demonstrated a leading role for mesenchymal factors in the differentiation of epithelial cell systems. These factors are highly specific because only organotypic mesenchyme appears to be able to induce appropriate differentiation. The factors responsible for differentiation induction have not been fully identified as yet.

Epithelial cell induction of stroma production

The bidirectional character of stroma-epithelium communication is exemplified by experiments demonstrating that epithelial cells elaborate factors which induce stromal cells (fibroblasts, myofibroblasts, endothelium) to proliferate and/or deposit extracellular matrix. Striking examples of this process are angiogenesis in a developing neoplasm (Furcht, 1986) and the desmoplastic reaction, which may occur in many types of carcinoma (Barsky and Gopalakrishna, 1987). Also in normal tissue development growth of the mesenchymal compartment (at least partly) depends on inducing factors from epithelial cells.

Epithelial-stromal interaction in carcinomas

As indicated above, the interface between stroma and epithelium is a site of dynamic action. This is especially true in malignant neoplasms, where at the tumor cell-stroma interface invasive growth, the hall-mark of malignancy and an essential prelude to metastasis, occurs. Theoretically, a whole cascade of events is required at this interface in the development of a metastasis. First of all, the neoplastic cells must acquire the potential to proteolytically degrade the basement membrane. Much research has focused on various aspects of tumor cell invasion and several proteolytic enzyme systems involved in this process have been identified (Tryggvason, 1989). Invasion implies, however, that cancer cells actively migrate into the surrounding mesenchymal compartment. This requires dissolution of intercellular and cell-matrix contacts. It might be envisioned, therefore, that in invasive cells the expression of integrins and of CAMs and cadherins is down-regulated (Mareel

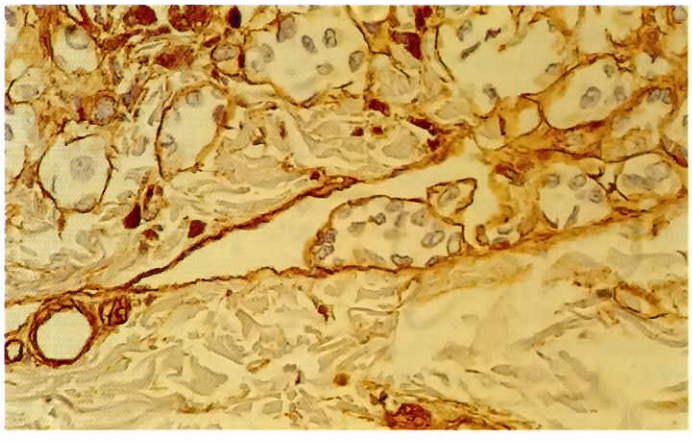

Fig. 2. Angioinvasive growth in a melanoma. *Note melanoma cells surrounded by a continuous layer of type IV collagen immunoreactive material in a capillary demarcated by a type IV collagen immunoreactive basement membrane (indirect immunoperoxidase; x120).*

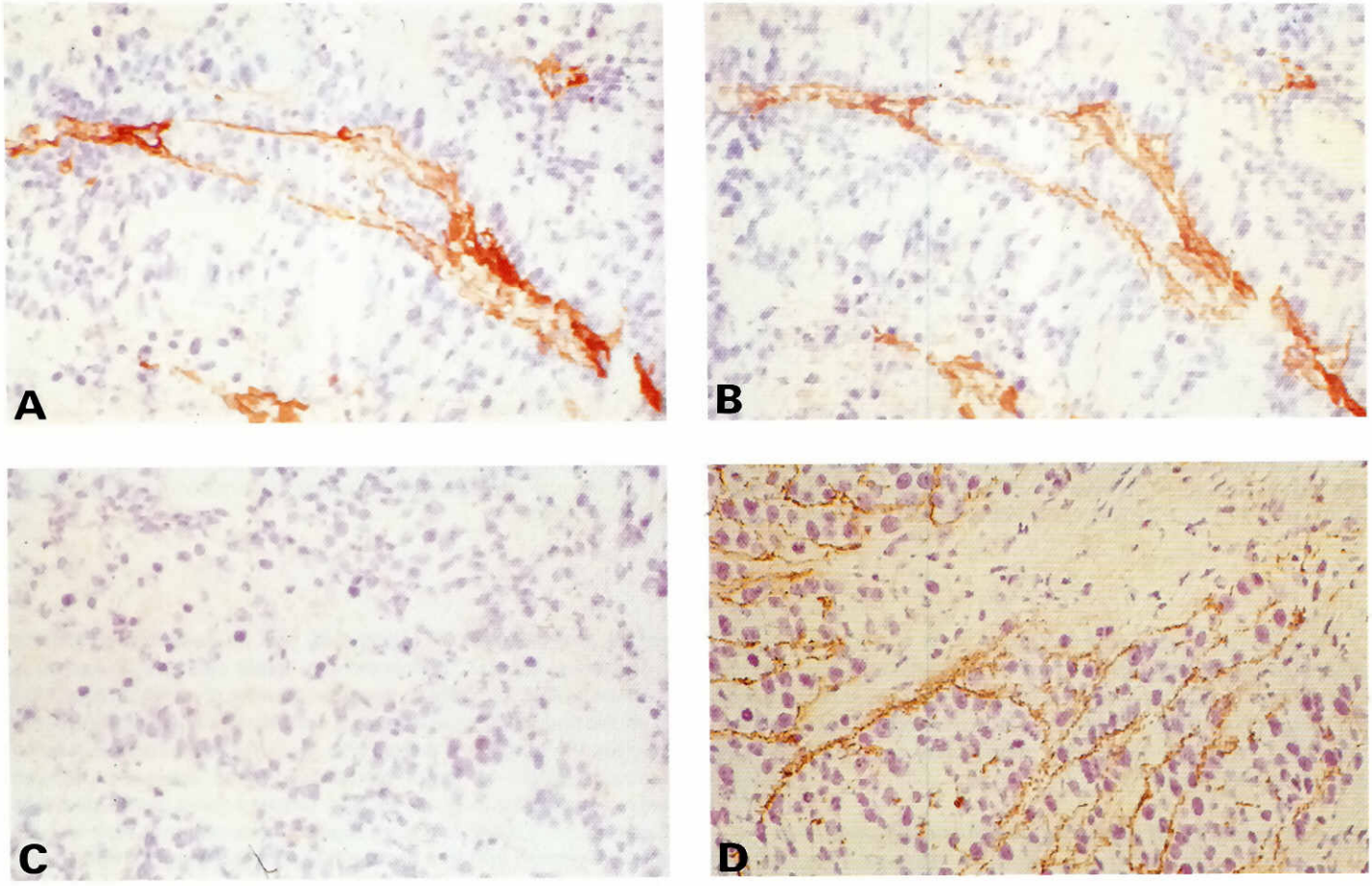

Fig. 3. Basement membrane deposition in xenografts of human colon cancer (A, B, C) and squamous cell carcinoma (D). *In xenografts of 5583 (human colonic adenocarcinoma) cells species cross reactive type IV collagen reactive antibodies detect basement membranes* **(A)** *which contain mouse epitopes* **(B)** *but not human epitopes* **(C)**. *In a xenograft of KB (human squamous cell carcinoma) cells, a human type IV collagen epitope is detected (A. rabbit anti-human type IV collagen antibody; B. rabbit anti-rat type IV collagen antibody, rat and mouse specific; C, D. mouse monoclonal anti-human type IV antibody, human specific; indirect immunoperoxidase; x130).*

et al., 1991). Migration factors will be required to induce migratory behavior. Tumor-specific migration factors have been identified (Nabeshima *et al.*, 1986). For the circulating cancer cells to home in on a potential metastatic site, cell surface adhesion molecules will be necessary. Up-regulation of the expression of CAMs and cadherins might be involved in this phase of metastasis development. For a metastasis to form, tumor stroma has to develop. Growth factors, such as b-FGF and TGFα, and also angiogenesis factors will be released from the tumor cells to this effect. For a metastasis to develop tissue architecture, integrins, CAMs and cadherins and the deposition of a basement membrane will be required. Therefore, up-regulation of the expression of these proteins may also be expected in the final stage of metastasis development. This theoretical sequence of events points to an important general principle. Increased or decreased expression of specific genes during invasion and metastasis might not be constitutive and irreversible but rather dynamic alterations in cell function. Up-regulation of the expression of proteolytic enzymes, decreased basement membrane production and down-regulation of E-cadherin expression may be temporary phenomena subject to modulation according to the phase of the development of a metastasis.

Studies on tumor cell-stromal interaction in colon cancer

In the last decade our group has been actively involved in studies on tumor-cell extracellular matrix interaction in human colon cancer in primary tumor tissue, in tissue culture and in xenograft models. Evidently, similar investigations have been performed extensively in a wide variety of other types of cancer. The general principles, however, can be illustrated in the colon cancer model.

Basement membrane degradation at the site of tumor cell invasion

In the normal colon the mucosal epithelium is outlined by a continuous and regular basement membrane. In the developing immune response, lymphocytes migrate from the lamina propria into the epithelium and back, which leads to circumscript holes in

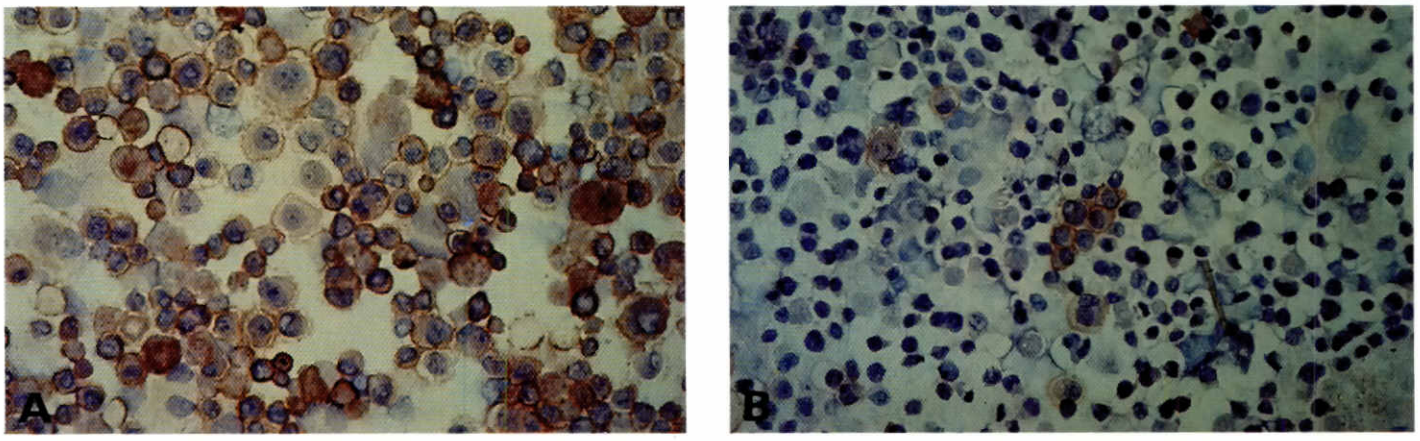

Fig. 4. VLA ($ß_1$ integrin) expression on H716 cells. (A) *α_2 chain immunoreactivity in a majority of the tumor cells.* **(B)** *α_3 chain immunoreactivity on scattered tumor cells (indirect immunoperoxidase; x250).*

the basement membrane especially in mucosa with lymphoid follicles (McClugage *et al.*, 1986). In inflammatory conditions a similar phenomenon is noted: where inflammatory cells affect the epithelium the basement membrane tends to be dissolved.

In hyperplastic polyps in the colon no basement membrane abnormalities are noted. In colonic adenomas, however, depending on the degree of epithelial (cytonuclear and architectural) atypia, discrete disruptions in the basement membrane can be found. This is especially seen in adenomas with severe atypia, regardless of the adenoma architecture (tubular or villous). In an area of invasive carcinoma in an adenoma basement membrane disruption is more striking (Fig. 1). These observations suggest that it might be possible to divide adenomas with severe atypia into a subgroup with and a subgroup without a tendency towards invasion. In view of the fact that all detected adenomas are excised, it is rather difficult to verify the validity of this assumption in human material.

In colonic adenocarcinomas a variable basement membrane pattern is seen, within as well as between tumors (Burtin *et al.*, 1983; Forster *et al.*, 1984, 1986; Daneker *et al.*, 1987; Havenith *et al.*, 1988). Within single tumors, in general a difference is found between the tumor center and the tumor periphery. In the tumor periphery invariably most of the tumor cell nests do not display any basement membrane material, reflecting active invasive growth. In the tumor center, however, basement membranes are deposited around the tumor cell groups to a variable extent. Some correlation seems to exist between tumor differentiation and basement membrane deposition, well differentiated tumors depositing more basement membrane material than poorly differentiated tumors. This correlation does not hold in all individual cases, however. Havenith *et al.* (1988) demonstrated that colon carcinomas with extensive deposition of basement membranes have a more favorable prognosis than tumors lacking this feature. This correlation was also found by other investigators (Forster *et al.*, 1984; Daneker *et al.*, 1987).

In the process of basement membrane dissolution at the invasive front of a colorectal carcinoma a variety of proteases is involved. The most specific of these is a group of collagenases with specificity for type IV collagen (Salo *et al.*, 1982). Several molecular variants of type IV collagenase have been identified (Tsuda *et al.*, 1988). Of some of these the gene has been cloned and characterized and antibodies have been generated. By immunohistochemistry the localization of this protease has been demonstrated in invasive carcinomas (Monteagudo *et al.*, 1990). This approach has been proposed as a possible means of distinguishing between invasive and non-invasive neoplasms. Also tumor grading according to the presence or absence of type IV collagen has been proposed.

Other proteases involved in basement membrane breakdown are trypsin and pepsin as well as the group of cathepsins, especially cathepsin B. Also the plasmin system is involved. Tissue plasminogen activator as well as plasminogen activator inhibitors and plasminogen activator receptor have been demonstrated on tumor cells as well as on tumor stroma. Plasmin is not involved in type IV collagen breakdown but it degrades laminin and fibronectin and activates other proteases by cleavage (Tryggvason, 1989).

Basement membrane deposition in primary and metastatic carcinomas

Initially, basement membrane penetration in malignant neoplasms was regarded as a result of mechanical forces, pressure building up in a confined tissue compartment due to volume increase, finally leading to rupture. It is now clear that at the tumor cell-stroma interface a dynamic process occurs which includes basement membrane dissolution — as outlined above — as well as basement membrane deposition. The pattern of occurrence of basement membranes in a malignant neoplasm appears to be the net result of this process of dissolution and deposition. A striking example of basement membrane deposition is seen in angioinvasive and in metastatic lesions. Obviously, tumor cells must have penetrated a basement membrane before entering the vascular system to embark on the process of metastasis, but nevertheless in intravascular cell nests and in metastases epithelial basement membranes can be readily detected (Fig. 2). This observation raises the question of which cells are responsible for the deposition of basement membrane material. In principle, stromal cells, as well as the tumor cells, could be involved. Xenotransplantation models of human cancer in nude mice have provided a means of studying the

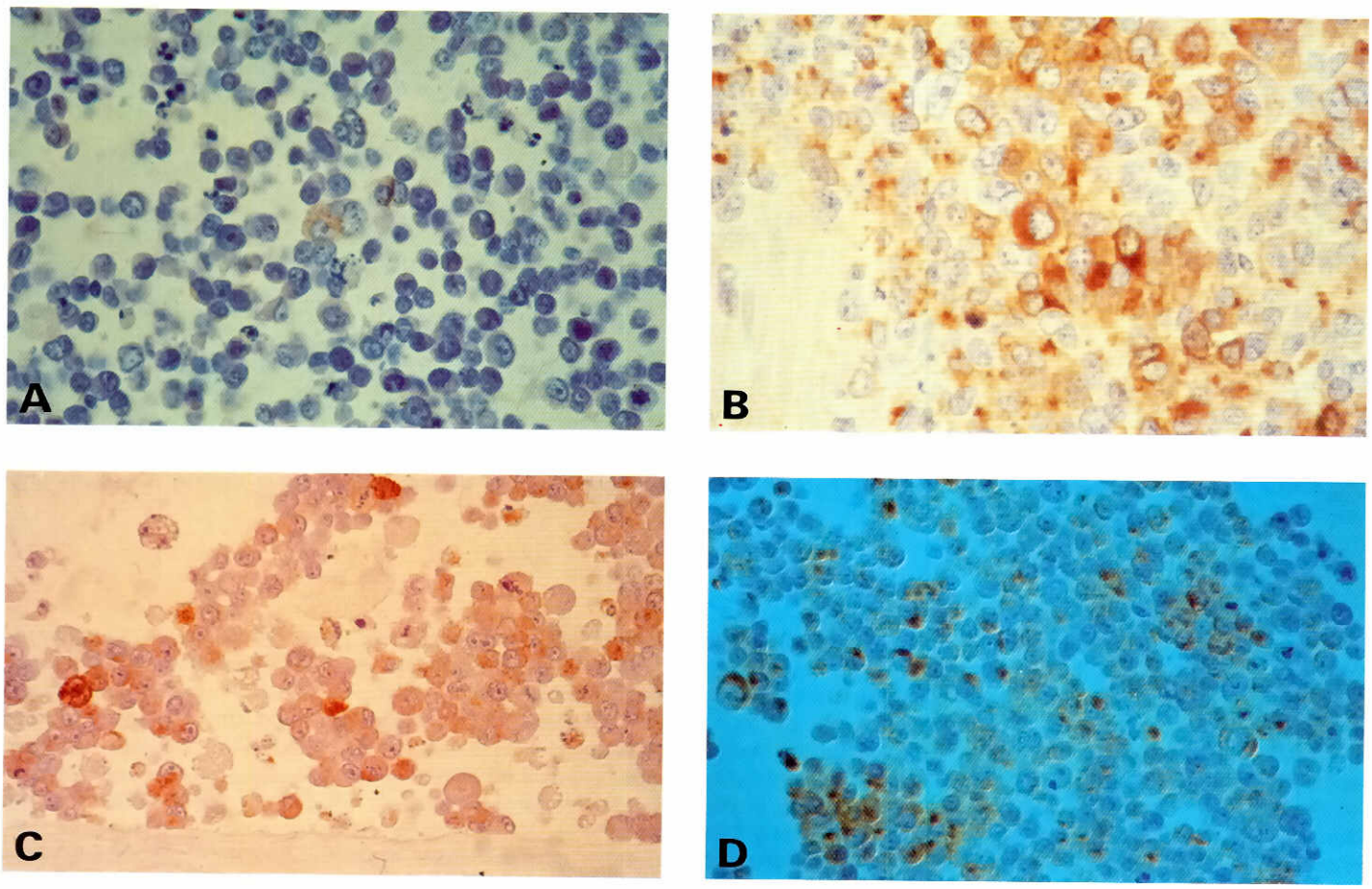

Fig. 5. Endocrine differentiation in H716 colonic adenocarcinoma cells as reflected in chromogranin A (CA) expression. (A) *H716 cells in vitro under standard conditions. Sporadically a tumor cell displays CA immunoreactivity.* **(B)** *H716 cells xenografted in a nude mouse. A majority of the tumor cells display CA immunoreactivity.* **(C)** *H716 cells cultured on amnion basement membrane. A majority of the cells express CA.* **(D)** *H716 cells cultured on collagen type IV in a medium supplemented with heparansulphate proteoglycan. Also under these circumstances a majority of the cells express CA (indirect immunoperoxidase; x250).*

origin of the basement membranes. It appeared feasible to prepare species specific (monoclonal or polyclonal) antibodies against basement membrane components. Using such reagents the origin of basement membrane material can be ascertained in xenografts. With this approach Damjanov *et al.* (1985) demonstrated that the laminin in basement membranes in xenografts of human hepatocellular carcinoma was of stromal (mouse) origin, whereas in xenografts of lung adenocarcinoma and yolk sac carcinoma, both producing laminin *in vitro*, the basement membranes contained epithelial (human) as well as stromal (mouse) elements. In our group Cleutjens *et al.* (1990) performed similar experiments using species specific antibodies against type IV collagen. In xenografts of human tumor cell lines which showed biosynthesis of basement membrane components *in vitro* (KB epidermoid carcinoma cells and WISH transformed amnion cells) the detected basement membranes contained a mouse (stromal) as well as a human (epithelial) component. In xenografts of cell lines which did not produce basement membrane components *in vitro* (HT 29 and 5583 E) the basement membrane appeared to be exclusively of mouse (stromal) origin (Fig. 3). These findings were corroborated by *in situ* hybridization experiments, using a cDNA probe to detect type IV collagen mRNA.

Our results underline the fact that neoplastic epithelial cells may retain their capacity to deposit basement membranes. The extent to which they do this seems to reflect the degree of differentiation of the neoplastic cells, poorly differentiated cells depositing little or no basement membrane material. It can also be argued that the tendency of tumor cells to be enveloped by basement membrane material reflects the host response to the neoplastic cells. This assumption is in line with our observations regarding the prognostic implications of basement membrane deposition in colorectal cancer (Havenith *et al.*, 1988) and also in bladder cancer (Schapers *et al.*, 1990).

Which factors are responsible for the induction of the deposition of basement membrane components by tumor stroma is not clear as yet. It is very likely that stromal myofibroblasts are the cells responsible for the deposition of this material. It seems reasonable to assume that in this process cell-matrix adhesion molecules, such

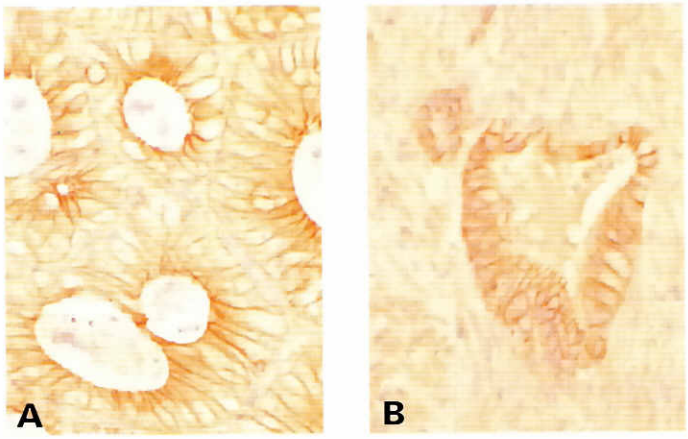

Fig. 6. E-cadherin immunoreactivity on colon adenoma and colon carcinoma. (A) *Intense expression on the lateral cell borders of columnar cells in a tubular adenoma.* **(B)** *Focal expression on colon carcinoma cells, invading the muscularis propria (indirect immunoperoxidase; x250).*

as integrins, as well as polypeptide growth factors, such as basic FGF or TGFß, are involved.

Induction of tumor cell differentiation by stromal components

In the last decade, increasing evidence has emerged supporting the active role of extracellular matrix components in the control of proliferation, differentiation and gene expression of normal and neoplastic cells. Studies in which fetal intestinal tissues were dissociated into mesenchymal and epithelial elements and reimplanted in syngeneic rodents have provided evidence for a leading influence of the mesenchyme on epithelial morphogenesis and differentiation (Yasugi and Mizuno, 1978; Haffen *et al.*, 1987). Interspecies transplantation experiments, using the chick-quail model, have provided additional evidence for a specific role for the mesenchyme in epithelial differentiation (Andrews, 1985). Kedinger *et al.* (1981, 1986) demonstrated that *in vitro* fetal gut mesenchyme induces differentiation in cultured endodermal and intestinal crypt cells, following similar experiments in the prostate by Cunha (1976). Evidence that mesenchymal elements can induce differentiation of epithelial cells has been provided for the colorectal carcinoma cell lines HT-29, CaCo2, LS 174T and HRA-19 (Haffen *et al.*, 1981; Fukamachi *et al.*, 1986; Del Bueno *et al.*, 1991). For CaCo2 and LS 174T it was shown that *in vivo* a high level of glandular differentiation is attained, contrasting with the lack of differentiation in standard *in vitro* conditions. Similar results were obtained for murine MAC 15 adenocarcinoma cells (Walling *et al.*, 1991). *In vitro*, differentiation could be induced in HT-29, CaCo2 and LS 174T by combining the cells with mesenchymal elements. Of special interest is the HRA-19 cell line in which not only glandular differentiation but also endocrine differentiation is induced *in vivo* (Kirkland, 1986). This has not been reproduced *in vitro*, however (Del Bueno *et al.*, 1991).

We have performed comparable experiments using the H716 cell line, originally described by Park *et al.* (1987). This cell line has been derived from a poorly differentiated cecal carcinoma and was shown to display endocrine characteristics, including neuroendocrine granules and dopa-d-carboxylase production. We observed under standard tissue culture conditions a limited level of endocrine differentiation in H716 cells as reflected in chromogranin immunoreactivity, which was remarkably augmented by xenotransplantation in nude mice. A plausible explantation for this difference would be that *in vivo* mesenchymal elements induce endocrine differentiation. Attempts were therefore made to create *in vitro* conditions which would also lead to increased endocrine differentiation. Culturing of the cells on different extracellular matrix components, including collagen types I and IV, laminin and fibronectin as well as culturing on denuded amnionic membranes were chosen. On purified extracellular matrix components H716 cells, which normally grow in suspension and do not adhere, specifically adhered only to type IV collagen. Immunohistochemically the $ß_1$ integrins VLA-2 and VLA-3, both collagen receptors, were detected on H716 cells (Fig. 4), which suggests that the type IV collagen binding is mediated by these integrins. In agreement with the lack of binding to laminin, the cells did not display VLA-6, a laminin receptor. Substrate adhesion to type IV collagen, however, did not induce endocrine differentiation. The amnionic membrane cultures displayed striking induction of endocrine differentiation, which proved that an intact extracellular matrix does contain differentiation-inducing substances. Strikingly, co-culturing with various types of fibroblasts also induced endocrine differentiation. H716 cells specifically adhered to these fibroblasts, without interposition of an extracellular matrix. Addition of 10 nM of b-FGF or of heparansulphate proteoglycan but not of TGFß *in vitro* induced endocrine differentiation (Fig. 5). These experiments prove that natural extracellular matrix contains factors which induce endocrine differentiation (De Bruïne *et al.*, 1993). One of these factors appears to be b-FGF, presumably produced by fibroblasts, in view of the observation that they also induced endocrine differentiation.

Cell-cell and cell-matrix adhesion molecules in colon cancer

Given the tendency of cancer cells to assume characteristic patterns of multicellular organization and the role of the extracellular matrix in inducing and maintaining that organization it is not surprising that CAMs and cell-matrix adhesion molecules, especially of the integrin type, occur abundantly on cancer cells (Koretz *et al.*, 1991; Shiozaki *et al.*, 1991). Elegant studies by Mareel and co-workers suggest a key role for E-cadherin (L-CAM) in the process of invasion and metastasis. Suppression of E-cadherin expression by transfection with an antisense gene or blocking of the function of the protein by monoclonal antibodies induced an invasive phenotype in previously non-invasive MDCK cells. Restitution of E-cadherin expression reversed the invasive phenotype (Behrens *et al.*, 1989; Vleminckx *et al.*, 1991). This observation prompted several investigators to study E-cadherin expression in human cancer, including colon cancer (Shiozaki *et al.*, 1991). Our own results, on a series of colon mucosa, adenoma and carcinoma specimens, confirm the intercellular expression of E-cadherin on epithelial cells in the normal mucosa. In adenomas this pattern appeared to be retained (Fig. 6A), although we found the overall level of E-cadherin expression to be decreased. Intensity of E-cadherin immunostaining gradually decreased towards the development of malignancy and in malignant neoplasms towards loss of differentiation. E-cadherin appeared to be expressed even in metastases. A difference between the level of E-cadherin expression in the center and the invasive periphery of colon carcinomas

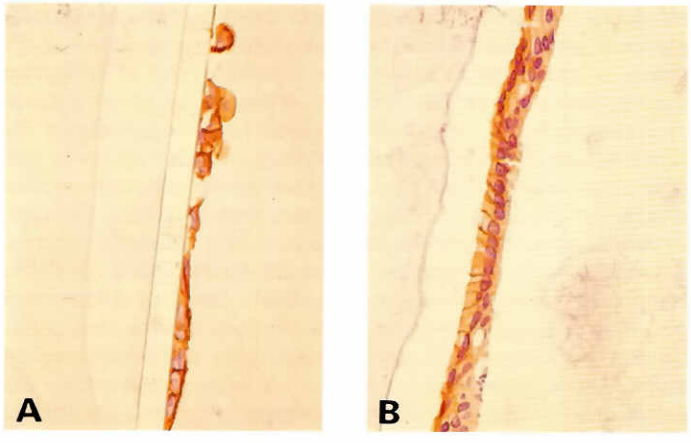

Fig. 7. Redistribution of VLA ($ß_1$ integrin) expression on differentiating HT-29 cells. (A) *α_3 chain expression circumferentially on undifferentiated HT-29 cells.* **(B)** *α_3 chain expression only on the lateral cell borders of differentiated HT-29 cells (immunoperoxidase; x250).*

was not found (Fig. 6B). These findings suggest that down-regulation of E-cadherin expression is a relatively early event in human colon carcinogenesis and does not appear to parallel the emergence of an invasive phenotype (Van der Wurff *et al.*, 1992).

In the induction and maintenance of the differentiated phenotype and three-dimensional organization of colon cancer, integrins have been found of significant importance. Pignatelli and Bodmer (1988, 1989, 1990) and Pignatelli *et al.* (1990) reported that loss of responsiveness of colon cancer cells to differentiating signals of collagen is paralleled by loss of an integrin type receptor. Hall *et al.* (1991) however reported that a clear relationship does not exist between differentiation and integrin expression in pancreatic carcinoma, and similar observations were reported in colorectal carcinomas by Koretz *et al.* (1991).

We studied integrin $ß_1$ chain and α_2, α_3 and α_6 chain distribution in colon mucosa and in colon adenomas and carcinomas (Flohil *et al.*, unpublished results). In line with the observations of Koretz *et al.* (1991), we found VLA-2 and VLA-3 to be distributed on the lateral surface of normal colonic epithelial cells. VLA-6 immunoreactivity was found on the basal plane of the cell, facing the basement membrane. In general, α chains were co-expressed with the $ß_1$ chain. In colonic adenomas and carcinomas a decreased level of VLA-2, VLA-3 and VLA-6 expression was noted. Especially in carcinomas heterogeneous $ß_1$ integrin expression occurred. In some specimens α and ß chains were no longer co-distributed. These findings closely correspond with those of Koretz *et al.* (1991), who also found a significant correlation between tumor stage (according to Dukes) and VLA-2 expression, VLA-2 being less expressed in Dukes' stages C and D.

We furthermore studied the expression of integrin α_2, α_3 and α_6 chains as well as $ß_1$ and $ß_4$ chains by immunohistochemistry on differentiated and undifferentiated HT-29 and CaCo2 cells, assuming that either the pattern of integrin expression or the distribution of integrins on the cell surface might change with differentiation. Also, integrin function was tested by adhesion experiments, using substrates coated with types I and IV collagen, laminin and fibronectin with or without prior incubation of the cells with anti-integrin antibodies.

By immunohistochemistry, α_2, α_3 and α_6 as well as $ß_1$ and $ß_4$ were located circumferentially on the surface of undifferentiated HT-29 cells. However, on differentiated cells the apical cell surface lacked integrin immunoreactivity (Fig. 7). Undifferentiated HT-29 and CaCo2 cells adhered to laminin, type IV collagen and fibronectin; in differentiated HT-29 cells laminin binding was reduced. Binding of CaCo2 and HT-29 cells could be blocked by anti-$ß_1$ antibodies.

These experiments indicate that in intestinal epithelial differentiation it is not the integrin expression as such but rather its topography on the cell surface that changes, and that in these cells α_2, α_6 and $ß_1$ integrin chains play an important role in cell-cell and cell-extracellular matrix adhesion.

Conclusions

The experiments described above illustrate the importance of tumor cell-extracellular matrix interactions in the development and behavior of carcinomas. The following general conclusions can be drawn.

The disruption of basement membranes in tumor invasion reflects a shift in basement membrane turnover, in the direction of increased degradation. The same tumor cells in a different environment, such as a developing metastasis, may return to basement membrane deposition.

Basement membranes in a neoplasm are deposited by stromal cells or by the concerted action of stromal and epithelial cells. Extensive basement membrane deposition is correlated with a favorable prognosis and reflects a high degree of tumor cell differentiation or a competent host reaction or both.

Tumor cell differentiation is modulated by extracellular matrix components, including growth factors such as b-FGF.

The cell surface expression of cell adhesion molecules and integrin receptors tends to be down-regulated in neoplastic cells. The observed alterations in the expression of these proteins may not be constitutive but subject to modulating factors from the environment, e.g. temporarily down-regulated during invasion but up-regulated during formation of a metastasis.

Acknowledgments

Expert technical assistance was provided by C. van Vroonhoven and E. v.d. Linden. The generous supply of anti-integrin antibodies by Dr. A. Sonneberg is gratefully acknowledged. Mrs. M. Bominaar typed the manuscript.

Fred Bosman first met Barry Pierce during a sabbatical leave spent in the Department of Pathology at the University of Colorado Health Sciences Center in Denver in 1977 and 1978. During this sabbatical he joined Paul Nakane's group, working on immunoelectronmicroscopical localization of nuclear antigens on chromosomes and of peptide hormone receptors on pituitary cells. The views of Barry Pierce on the biology of cancer and on the parallels between oncogenesis and ontogenesis profoundly influenced his own scientific development. The early work on basement membranes in germ cell cancer in Barry Pierce's group led him to explore the role of base membrane components in cancer differentiation and the use of basement membrane immunohistochemistry in cancer diagnosis. Through the years his ties with the Denver Department remained and his personal relationship with Barry and Donna Pierce deepened, which resulted in close friendship between the Pierce and Bosman families.

References

ANDREWS, A. (1985). Developmental relationship of neuroendocrine cells. *Biomed. Res. 6:* 191-196.

BARSKY, S.H. and GOPALAKRISHNA, R. (1987). An experimental model for studying the desmoplastic response to tumor invasion. *Cancer Lett. 35:* 271-279.

BEHRENS, J., MAREEL, M.M., VAN ROY, F. and BIRCHMEIER, W. (1989). Dissecting tumor cell invasion; epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. *J. Cell. Biol. 108:* 2435-2447.

BURTIN, P., CHAVANEL, G. and FOIDART, J.M. (1983). Immunofluorescence study of the antigens of the basement membrane and the peritumoral stroma in human colonic adenocarcinomas. *Ann. NY Acad. Sci. 420:* 229-236.

CLEUTJENS, J.P.M., HAVENITH, M.G., BEEK, C., VALLINGA, M., KATE, J.T. and BOSMAN, F.T. (1990). Origin of basement membrane type IV collagen in xenografted human epithelial tumor cell lines. *Am. J. Pathol. 136:* 1165-1172.

CUNHA, G.R. (1976). Epithelial-stromal interactions in development of the urogenital tract. *Int. Rev. Cytol. 47:* 137-194.

DAMJANOV, I., DAMJANOV, N., KNOWLESS, B.B. and ENGVALL, E. (1985). Origin of laminin in the extracellular matrix of human tumour xenografts in nude mice. *Virchows Arch. B Cell Pathol. 49:* 45-52.

DANEKER, G.W., MERCURIO, A.M., GUERRA, L., WOLF, B., SALEM, R.R., BAGLI, D.J. and STEELE, G.D., Jr. (1987). Laminin expression in colorectal carcinomas varying in degree and differentiation. *Arch. Surg. 122:* 1470-1474.

DE BRUÏNE, A.P., DINJENS, W.N.M., VAN DER LINDEN, E.P.M., PYLS, M. MOERKERK, P. and BOSMAN, F.T. (1992). Extracellular matrix components induce endocrine differentiation *in vitro* in NCI-H716 cells. *Am. J. Pathol.* (In press).

DEL BUENO, R., PIGNATELLI, M. and HALL, P.A. (1991). Control of differentiation in a rectal adenocarcinoma cell line: the role of diffusable and cell-associated factors. *J. Pathol. 164:* 59-66.

FORSTER, S.J., TALBOT, I.C. and CRITCHLEY, D.R. (1984). Laminin and fibronectin in rectal adenocarcinoma: relationship to tumour grade, stage and metastasis. *Br. J. Cancer 50:* 51-61.

FORSTER, S.J., TALBOT, I.C., CLAYTON, D.G. and CRITCHLEY, D.R. (1986). Tumour basement membrane laminin in adenocarcinoma of rectum: an immunohistochemical study of biological and clinical significance. *Int. J. Cancer 37:* 813-817.

FUKAMACHI, H., MIZUNO, T. and KIM, Y.S. (1986). Morphogenesis of human colon cancer cells with fetal rat mesenchymes in organ culture. *Experientia 42:* 312-315.

FURCHT, L.T. (1986). Critical factors controlling angiogenesis: cell products, cell matrix, and growth factors. *Lab. Invest. 55:* 505-509.

HAFFEN, K., KEDINGER, M. and SIMON-ASSMAN, P.M. (1987). Review. Mesenchyme dependent differentiation of epithelial progenitor cells in the gut. *J. Pediatr. Gastroenterol. Nutr. 6:* 14-23.

HAFFEN, K., SIMON-ASSMAN, P.M., GRENIER, J.F. and ZWEIBAUM, A. (1981). Effet permissif des cellules cancereuses coliques humanes (HT-29 et CaCo-2). *C.R. Acad. Sci. Paris 293:* 807-812.

HALL, P.A., COATES, P., LEMOINE, N.R. and HORTON, M.A. (1991). Characterization of integrin chains in normal and neoplastic human pancreas. *J. Pathol. 165:* 33-41.

HAVENITH, M., ARENDS, J.W., SIMON, R., VOLOVICS, A., WIGGERS, T. and BOSMAN, F.T. (1988). Type IV collagen immunoreactivity in colorectal cancer. Prognostic value of basement membranes deposition. *Cancer 62:* 2207-2211.

HEMLER, M.E. (1990). VLA proteins in the integrin family: structures, functions and their role on leucocytes. *Annu. Rev. Immunol. 8:* 365-400.

KEDINGER, M., SIMON, P.M., GRENIER, J.F. and HAFFEN, K. (1981). Role of epithelial mesenchyme interactions in the ontogenesis of brush border enzymes. *Dev. Biol. 86:* 339-347.

KEDINGER, M., SIMON-ASSMAN, P.M., LACROIX, B., MARXER, A., HAURI, H.P. and HAFFEN K. (1986). Fetal gut mesenchyme induces differentiation of cultured intestinal endodermal and crypt cells. *Dev. Biol. 113:* 473-483.

KIRKLAND, S.C. (1986). Endocrine differentiation by a human rectal adenocarcinoma cell line (HRA-19). *Differentiation 33:* 148-155.

KORETZ, K., SCHLAG, P., BOUMSELL, L. and MÖLLER, P. (1991). Expression of VLA-α_2, VLA-α_6, and VLA-β_1 chains in normal mucosa and adenomas of the colon, and in colon carcinomas and their liver metastases. *Am. J. Pathol. 138:* 741-750.

LUNSTRUM, G.P., KUO, H.J., ROSENBAUM, L.M., KEENE, D.R., GRANVILLE, R.W. and SAKAI, L.Y. (1987). Anchoring fibrils contain the carboxyl-terminal globular domain of type VII procollagen but lack the amino-terminal globular domain. *J. Biol. Chem 262:* 13706-13712.

MAFUNE, K., RAVIKUMAR, T.S., WONG, J.M., YOW, H., CHEN, L.B. and STEELE, G.D., Jr. (1990). Expression of a M_r32,000 laminin-binding protein messenger RNA in human colon carcinoma correlates with disease progression. *Cancer Res. 50:* 3888-3891.

MAREEL, M., DE BAETSELIER, P. and VAN ROY, F. (1991). *Mechanisms of Invasion and Metastasis.* CRS Press, Boca Raton.

MARTINEZ-HERNANDEZ, A. and AMENTA, P. (1983). The basement membrane in pathology. *Lab. Invest. 48:* 656-677.

McCLUGAGE, S.G., LOW, F.N. and ZIMNY, M.L. (1986). Porosity of the basement membrane overlying Peyer's patches in rats and monkeys. *Gastroenterology 91:* 1128-1133.

MONTEAGUDO, C., MERINO, M.J., SAN-JUNA, J., LIOTTO, L.A. and STETTLER-STEVENSON, W. (1990). Immunohistochemical distribution of type IV collagenase in normal, benign and malignant breast tissue. *Am. J. Pathol. 136:* 585-592.

NABESHIMA, K., KATAOKA, H. and KOONO, M. (1986). Enhanced migration of tumor cells in response to collagen degradation products and tumor cell collagenolytic activity. *Invasion Metastasis 6:* 270-286.

PARK, J.G., OIE, H.K., SUGARBAKER, P.H., HENSLEE, J.G., CHEN, T.R., JOHNSON, B.E. and GAZDAR, A. (1987). Characteristics of cell lines established from human colorectal carcinoma. *Cancer Res. 47:* 6710-6718.

PIGNATELLI, M. and BODMER, W.F. (1988). Genetics and biochemistry of collagen binding-triggered glandular differentiation in a human colon carcinoma cell line. *Proc. Natl. Acad. Sci. USA 85:* 5561-5565.

PIGNATELLI, M. and BODMER, W.F. (1989). Integrin-receptor-mediated differentiation and growth inhibition are enhanced by transforming growth factor-ß in colorectal tumour cells grown in collagen gel. *Int. J. Cancer 44:* 518-523.

PIGNATELLI, M. and BODMER, W.F. (1990). Integrin cell adhesion molecules and colorectal cancer. *J. Pathol. 162:* 95-97.

PIGNATELLI, M., SMITH, M.E.F. and BODMER, W.F. (1990). Low expression of collagen receptors in moderate and poorly differentiated colorectal adenocarcinomas. *Br. J. Cancer 61:* 636-638.

REDDI, A.S. (1985). Metabolism of glomerular membrane in normal, hypophysectomized, and growth-hormone-treated diabetic rats. *Exp. Mol. Pathol. 43:* 196-208.

SALO, T., LIOTTA, L.A., KESKI-OJA, J., TURPEENNIEMI-HUJANEN, T. and TRYGGVASON, K. (1982). Secretion of basement membrane collagen degrading enzyme and plasminogen activator by transformed cells — role in metastasis. *Int. J. Cancer 30:* 669.

SASAKI, M., KLEINMAN, H.K., HUBER, H., DEUTZMAN, R. and YAMADA, Y. (1988). Laminin, a multidomain protein. *J. Biol. Chem. 263:* 16536-16544.

SCHAPERS, R.F.M., PAUWELS, R.P.E., HAVENITH, M.G., SMEETS, A.W.B.G., VAN DEN BRANDT, P.A. and BOSMAN, F.T. (1990). Prognostic significance of type IV collagen and laminin immunoreactivity in urothelial carcinomas of the bladder. *Cancer 66:* 2583-2588.

SHIOZAKI, H., TAHARA, H., OKA, H., MIYATA, M., KOBAYASHI, K., TAMURA, S., IIHARA, K., DOKI, Y., HIRANO, S., TAKEICHI, M. and MORI, T. (1991). Expression of immunoreactive E-cadherin adhesion molecules in human cancers. *Am. J. Pathol. 139:* 17-23.

SIMON-ASSMAN, P., BOUZIGES, F., ARNOLD, C., HAFFEN, K. and KEDINGER, M. (1988). Epithelial-mesenchyme interactions in the production of basement membrane components in the gut. *Development 102:* 339-347.

TAKEICHI, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science 251:* 1451-1455.

TRYGGVASON, K. (1989). Extracellular matrix and its enzymatic degradation in tumor invasion. In *Influence of Tumor Development on the Host* (Ed. L.A. Liotta). Kluwer Academic Publishers, Dordrecht, pp. 72-83.

TSUDA, M., YAMAGISHI, Y. and KATSUMIMA, T. (1988). High molecular mass type IV collagen-specific metalloprotease from human carcinoma tissue. *FEBS Lett. 232:* 140-144.

VAN DER WURFF, A.A.M., TEN KATE, J., VAN DER LINDEN, E.P.M., DINJENS, W.N.M., ARENDS, J.W. and BOSMAN, F.T. (1992). L-CAM expression in normal, premalignant and malignant colonic mucosa. *J. Pathol. 168:* 287-291.

VLEMINCKX, K., VAKAET, L. Jr., MAREEL, M.M., FIERS, W. and VAN ROY, F. (1991). Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell 66:* 107-119.

WALLING, J.M., BLACKMORE, M., HICKMAN, J.A. and TOWNSEND, K.M.S. (1991). Role of the extracellular matrix on the growth and differentiated phenotype of murine colonic adenocarcinoma cells *in vitro*. *Int. J. Cancer 47:* 776-783.

YASUGI, S. and MIZUNO, T. (1978). Differentiation of the digestive tract epithelium under the influence of the heterologous mesenchyme of the digestive tract in the bird embryo. *Dev. Growth Differ. 20:* 261-267.