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We have used a transgenic mouse model system to investigate the role of integrin defects in breast cancer progression. Transgenic mice carrying an activated form of the N-*ras* oncogene under the control of the Mammary Tumor Virus Long Terminal Repeat promoter develop mammary carcinomas with a high frequency during the first few months of their life. The $\alpha 6\beta 4$ and, to a minor extent, the $\alpha 2\beta 1$ integrin were found to be upregulated and diffusely distributed at the tumor cell surface in the primary lesions of these mice. These events were accompanied by a loss of laminin staining indicative of defective basement membrane deposition. The $\alpha 3\beta 1$ integrin was diffusely distributed, but not upregulated. Since transfection of N-ras into cultured murine breast epithelial cells did not produce the changes in integrin expression detected *in vivo*, it is likely that these changes occur as a result of tumor progression independently of a direct action of N-ras. The above described studies indicate that the expression, and possibly the function, of several integrins involved in adhesion to the basement membrane is altered during the *in vivo* progression of breast cancer in the N-ras transgenic mouse model.

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Filippo Giancotti 1/2/1996

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INTRODUCTION

Cell-matrix interactions are likely to play an important role in breast tumorigenesis. Most human breast cancers arise from the transformation of ductal epithelial cells (1-3). Normal ductal epithelial cells rest on a basement membrane, to which they adhere tightly (2). The adhesion of normal breast epithelial cells to the basement membrane is thought to be important for the organization of the cytoskeleton and the consequent establishment of polarity. In addition, recent results indicate that normal breast epithelial cells receive signals from the basement membrane and these signals help them maintain a differentiated phenotype (4). When compared to normal cells, breast carcinoma cells show a defective interaction with the basement membrane. First, like most carcinoma cells, they fail to assemble basement membrane components in an organized extracellular matrix, both *in vivo* and *in vitro* (5, 6), and show cytoskeletal defects (7). Second, in contrast to normal breast epithelial cells, carcinoma cells do not arrest their growth when placed in a reconstituted basement membrane gel (6). It is important to understand the molecular basis of these phenomena because they are likely to contribute to the ability of breast carcinoma cells to detach from the original tumor and invade adjacent tissues.

The molecular characterization of integrins provides a unique opportunity to examine the role of cell-matrix interactions in breast cancer. The integrins are a large family of adhesion receptors which bind to extracellular matrix components and, in some cases, to counter-receptors on other cells (8). They consists of two distinct membrane-spanning subunits, α and β. At present we know of at least 9 homologous β subunits and 15 α subunits which can combine to form 21 receptors with distinct ligand binding specificities. Both the α and the β subunit (each ca. 140-200 kD m.w.) have a large extracellular portion, a transmembrane segment, and a short cytoplasmic domain. A notable exception is the β_4 subunit that has a large cytoplasmic domain. While the extracellular N-termini of α and β subunits associate to form the ligand binding pocket, the cytoplasmic domains of integrins interact with intracellular molecules.

The binding of integrins to extracellular matrix components promotes cell adhesion or migration, but ligation of integrins also results in intracellular signals which influence proliferation and differentiation (9). While contact with extracellular matrix components is required for the progression of normal cells through the cell cycle, a phenomenon called anchorage dependence, strong adhesion to an organized extracellular matrix seems to be able to limit cell proliferation (10) and promote differentiation (4). The ability of integrins to modulate gene expression may help to explain the effects that the extracellular matrix has on proliferation and differentiation. The mechanisms by which integrins affect gene regulation are not completely understood, but likely depend on the ability of the cytoplasmic domains of integrins to interact both with the cytoskeleton (11) and with signaling molecules, such as Focal Adhesion Kinase (FAK) (12).

Neoplastic cells are characterized by a number of adhesion abnormalities which may explain their ability to grow independently of the positive and negative control signals originating from the extracellular matrix (13). Virally transformed fibroblasts have a more rounded morphology in culture than their non-transformed counterparts. In addition, they often lack a cell surface fibronectin-containing pericellular matrix (14). The defective fibronectin matrix of transformed fibroblasts may only partially be attributed to either decreased biosynthesis or increased proteolytic degradation of fibronectin, since the fibronectin secreted by transformed cells is regularly incorporated in the extracellular matrix by normal cells (15). This suggests that transformed cells can not retain at their surface the fibronectin they produce, perhaps because of a defect in the integrin receptors. Several observations indicate that the expression and function of integrins are altered in neoplastic fibroblasts. While in normal fibroblasts the β_1 integrins, which include the $\alpha_5\beta_1$ fibronectin receptor, are clustered in focal adhesions (16-18), transformed fibroblasts lack such structures and their β_1 integrins are found diffusely distributed over the cell surface (18, 19). In addition, in fibroblasts transformed by tyrosine kinase oncogenes the β_1 subunit is found to be partially phosphorylated on a tyrosine residue (20), a phenomenon which may reduce its ability to interact with the cytoskeleton (21). Finally, the expression of $\alpha_5\beta_1$ and of another β_1 integrin, probably $\alpha_1\beta_1$, is suppressed in fibroblasts transformed by oncogenic viruses (22).

We have tested the hypothesis that changes in the level of expression or function of the $\alpha_5\beta_1$ fibronectin receptor contribute to the adhesive abnormalities of transformed fibroblasts by overexpressing this integrin in Chinese hamster ovary (CHO) cells (10). The CHO cells have a transformed morphology, deposit little fibronectin in their pericellular matrix and are tumorigenic in vivo. As a result of the $\alpha_5\beta_1$ overexpression, the CHO cells accumulated a fibronectin matrix and became less migratory. These results, which are described in more detail in Preliminary Studies, indicate an inverse correlation between matrix assembly of fibronectin and cell migration and suggest that the loss of fibronectin matrix and the increased invasive ability of transformed fibroblasts can be both brought about by a reduced expression or function of $\alpha_5\beta_1$. Interestingly, the CHO cells overexpressing $\alpha_5\beta_1$ were also found to be more anchorage dependent than the controls and were not able to form subcutaneous tumors in nude mice. K562 leukemia cells selected for high level expression of $\alpha_5\beta_1$ show a similar normalization of growth properties (23). Conversely, CHO cells selected for their low levels of $\alpha_5\beta_1$ expression are more tumorigenic than unselected cells (24). Thus, it appears that changes in the level of expression or activity of certain integrins may not only be responsible for the adhesive defects of neoplastic cells but may also contribute to their unregulated growth. Taken together, these observations suggest that the role of integrins in tumorigenesis is twofold: first, integrins mediate stable adhesion or migration onto extracellular matrix components and changes in their level of expression and function may, therefore, contribute to

tumor invasion. Second, integrins transmit signals from the extracellular matrix to the cell interior and these signals affect cellular growth and differentiation. Therefore changes in integrins may contribute to the unrestrained growth and lack of differentiation of neoplastic cells.

Although the adhesive phenotype of breast carcinoma cells is less well known than that of neoplastic fibroblasts, certain rules learned from the analysis of virally transformed fibroblasts seem to also apply to these cells. For example, breast carcinoma cells fail to assemble basement membrane components in an organized extracellular matrix (5, 6) and show enhanced ability to grow when confronted with a reconstituted basement membrane gel (6). Immunohistochemical studies have indicated that the $\alpha_2\beta_1$ collagen/laminin receptor, the $\alpha_5\beta_1$ fibronectin receptor and the $\alpha_6\beta_4$ integrin, a receptor for various forms of laminin (unpublished results of E. Ruoslahti's and A. Sonnenberg's laboratories), are greatly reduced in human carcinomas of the breast (25-27). In addition, while integrins are generally polarized at the basal or baso-lateral surface in normal breast epithelium, the residual integrins expressed in breast carcinoma cells are diffusely distributed over the cell surface (25-27). It is our hypothesis that these phenomena contribute to the ability of breast carcinoma cells to detach from the original tumor and invade the adjacent tissues.

BODY

We have focused on establishing a transgenic mouse model system in which to investigate the role of integrin defects in breast cancer progression. To this end, we have examined transgenic mice carrying either an activated or a normal form of the N-*ras* oncogene under the control of the Mammary Tumor Virus Long Terminal Repeat (MMTV-LTR) promoter. These mice, similarly to mice carrying activated forms of the H-*ras* or *neu* oncogenes, develop mammary carcinomas with a high frequency during the first few months of their life (28, 29, 30; R. Mangues & A. Pellicer, Department of Pathology, N.Y.U. School of Medicine, unpublished results). The tumors which develop often consist of areas of different level of histological differentiation and thus can provide an insight to the process of primary breast tumor progression.

A) Immunohistochemical Analysis of Integrin Expression in Normal Murine Breast Tissue and Breast Tumors from a Point-Mutated N-ras Transgenic Mouse Line.

Tissue from normal murine lactating and N-ras oncogenic breast tumors were immediately embedded in OCT and frozen on dry ice and then stored at -80^0 C. Sections, 7 μm, were cut on a cryostat and placed on TES treated slides. The slides were briefly air-dried and then desiccated over night at 4^0 C. The sections were then fixed in ice cold acetone for 10 minutes and then allowed to air dry. H&E staining or immunohistochemical staining was then performed. For immunohistochemical staining, the sections were hydrated in PBS for 20 minutes at

room temperature. The sections were then blocked with PBS/10% normal goat serum for 1 hour in a humidified chamber. The sections were then incubated with the primary antibody solution (anti-integrin antibody in PBS-3% BSA) over night in a humidified chamber at 4^0 C. The slides were washed 3 times in PBS. For double labeling, an anti-laminin antibody solution, also in PBS-3% BSA, was applied for 1 hour at room temperature. Following washes with PBS, secondary antibody solutions with fluorescent-labeled antibodies were then applied for 1 hour each at room temperature. The slides were then mounted and examined by fluorescent microscopy.

Normal murine breast demonstrated laminin staining along the basement membranes of both inter- and intra-lobular ducts as well as those of alveoli. The α_6, β_4 and β_1 subunit were found to colocalize with laminin at the basement membrane junction. The α_3 subunit staining was predominant along the basement membrane of the ducts with less prominent alveolar basal staining. The α_2 subunit staining was similar to that for α_3 but less intense. There was no significant staining above background levels in the ducts for either α_5 or α_v. Discontinuous α_v staining could be detected at the myoepithelial basement membrane.

Oncogenic ras murine breast tumors displayed a significant loss of laminin staining. The α_6 and β_4 subunits were over-expressed, but lacked polarization; some basal staining could be seen in better differentiated tumor areas. The β_1 staining was similarly no longer polarized, but it was not upregulated. The α_2 and α_3 subunits were also diffusely expressed in the tumors with an apparent increased intensity of α_2 staining. The α_5 and α_v subunits were not expressed in the tumors.

B) Effect of Ras on Integrin Expression in a Murine Breast Cell Line.

To determine if the changes in integrin expression *in vivo* were a direct result of ras or due to other genetic changes which occur during tumor progression, the effect of the expression of the N-ras oncogene on integrin expression in abnormal murine breast cell line was investigated. The heterogeneous murine breast cell line NMuNg was dilutionally cloned to isolate an epithelial cell line with an integrin repertoire similar to that of normal breast epithelium. The dilutional clone was stably transfected with a cDNA encoding the N-ras oncogene and the neomycin resistance selection marker. Control cell lines were transfected with the neomycin resistance gene only. Positive clones expressing high levels of N-ras were selected after soft agar subcloning.

Cell surface labeling and immunoprecipitation analysis indicated that the breast epithelial cell lines acutely transformed by N-ras expressed an integrin repertoire indistinguishable from that of control untransformed cell lines. Moreover the level of expression of individual integrin subunits in N-ras expressing cell lines was unchanged as compared to the controls.

CONCLUSIONS

The above described studies indicate that the expression, and possibly the function, of several integrins involved in adhesion to the basement membrane is altered during the *in vivo* progression of breast cancer in the N-ras trangenic mouse model. The $\alpha_6\beta_4$ and, to a minor extent, the $\alpha_2\beta_1$ integrin are upregulated and diffusely distributed at the tumor cell surface in the primary lesions. These events are accompanied by a loss of laminin staining indicative of defective basement membrane deposition. The $\alpha_3\beta_1$ integrin is diffusely distributed, but not upregulated. Since transfection of N-ras into cultured murine breast epithelial cells does not produce the changes in integrin expression detected *in vivo*, it is likely that these changes occur as a result of tumor progression independently of a direct action of N-ras.

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