Prognostic Value of α6β4 Integrin Expression in Breast Carcinomas Is Affected by Laminin Production from Tumor Cells[1]

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ABSTRACT

Immunocytochemical analysis of breast carcinoma specimens for α6β4 integrin expression and other different pathobiological markers revealed β4 integrin subunit expression in 36 of 80 cases analyzed and a significant association only with α6 integrin subunit expression ($P < 0.01$) and laminin production ($P = 0.01$) by tumor cells. Survival analysis indicated that β4 and α6 expression are associated with poor prognosis ($P = 0.02$), whereas laminin production showed only borderline association ($P = 0.06$). However, analysis of disease outcome in relation to expression of both α6β4 and laminin indicated best outcomes for patients with tumors producing laminin but not expressing α6β4 integrin, whereas worst outcomes were observed for α6β4- and laminin-positive tumors, indicating that α6β4 expression was associated with prognosis, mainly in the laminin-producing tumor subset. These data indicate that the prognostic value of α6β4 integrin expression is affected by laminin production from tumor cells and suggest that interaction between these two molecules mediates distinct signals that are important for tumor progression.

INTRODUCTION

The α6β4 integrin, corresponding to the TSP-180 tumor-associated complex that was described in mouse mammary tumor cells and human carcinoma cell lines (1–3), is the receptor for various laminin isoforms (4, 5) and is mainly expressed in cell types that interact with laminin, *i.e.*, epithelial (6), endothelial (7, 8), and Schwann cells (9). Moreover, α6β4, by virtue of a specific tyrosine activation motif in the β4 tail, plays a crucial role in the assembly of hemidesmosomes (10), the junctions connecting the basal cells of stratified and complex epithelia to the basement membrane (11). The basal polarization of this molecular complex in normal epithelia results in its gradual loss in various solid tumors (12–16). The role of the α6β4 integrin in tumor progression is still unclear. Indeed, several studies indicate that α6β4 is overexpressed in squamous carcinomas of lung, skin, oral cavity, and cervix (14, 15, 17, 18), whereas it is down-regulated in adenocarcinomas derived from epithelial tissues that normally express high levels of this integrin, *e.g.*, adenocarcinomas of breast and prostate (19–21). Recent *in vitro* studies indicate that α6β4, which does not itself have an intracellular catalytic domain, is physically and functionally associated with cytoplasmic tyrosine kinases (7, 22) and that laminin binding to this integrin activates the associated kinase and consequently tyrosine phosphorylation of the β4 subunit cytoplasmic domain. Tyrosine phosphorylation motifs in the β4 tail, distinct from those involved in the assembly of hemidesmosomes, mediate the recruitment of Shc/Grb2 and may link the integrin to ras signaling pathways (10). Because the mitogen-activated protein kinase pathway activated by ras has been implicated in controlling proliferation or differentiation depending on the cellular context (23), the linkage of α6β4 to ras may explain the effects of laminins on morphogenesis and tumor progression. Recently, α6β4 overexpression in rectal carcinoma cells was found to induce $p21^{WAF1/CIP1}$ expression with consequent cell growth arrest but also with increased invasiveness (24). These contrasting results support the hypothesis that α6β4 is linked to a growth suppression pathway and that laminin binding reverses this connection and couples the integrin through Shc and Grb2 to ras with consequent cell proliferation and/or invasion. On the basis of these *in vivo* and *in vitro* studies, we analyzed breast carcinomas for expression of the α6β4 integrin and other pathobiological markers and the prognostic value of the α6β4 in relation to the laminin produced by the tumor cells.

PATIENTS AND METHODS

Patients. The study included 80 patients who were surgically treated at Istituto Nazionale Tumori (Milan, Italy) from 1988 to 1990: 51% showed no lymph node involvement and 49% presented a tumor that was ≤2 cm. Surgical treatment consisted of radical or modified radical mastectomy and axillary dissection. Histologically node-positive patients received post-surgical adjuvant treatment.

Immunocytochemical Staining. Frozen sections fixed in acetone were stained by the immunoperoxidase method using the avidin-biotin complex kit (Vector Laboratories, Burlingame, CA), and reactivity was detected with the following MAbs[3]: DO7, directed against p53 protein (Ylem-Novocastra, New Castle, United Kingdom); clone 124, directed against bcl-2 product (DAKO A/S, Glostrup, Denmark); MGR1, directed against the EGFR (25); MGR2, directed against $p185^{HER2}$ (26); MAR6, directed against

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[3] The abbreviations used are: MAb, monoclonal antibody; EGFR, epidermal growth factor receptor.

Table 1 Analysis of association between β4 integrin subunit expression and other evaluated parameters

Parameter	Category	No. of β4 + cases/no. of tested cases	%	*P*
Size	≤2 cm	23/45	51	
	>2 cm	12/34	35	
Node	+	21/44	48	
	−	15/36	42	
Grade	I–II	28/59	47	
	III	8/20	40	
ER[a]	+	26/62	42	
	−	7/12	58	
PGR	+	21/49	43	
	−	12/25	48	
LI	+	18/35	55	
	−	7/15	47	
$p185^{HER2}$	+	13/29	45	
	−	21/47	45	
EGFR	+	8/17	47	
	−	26/59	42	
p53	+	8/11	73	
	−	28/65	43	
bcl-2	+	21/40	52	
	−	10/27	37	
α6	+	32/34	94	<0.01
	−	3/45	7	
LN	+	18/27	67	0.01
	−	14/40	35	

[a] ER, estrogen receptor; PGR, progesterone receptor; LI, lymphoid infiltration; LN, laminin.

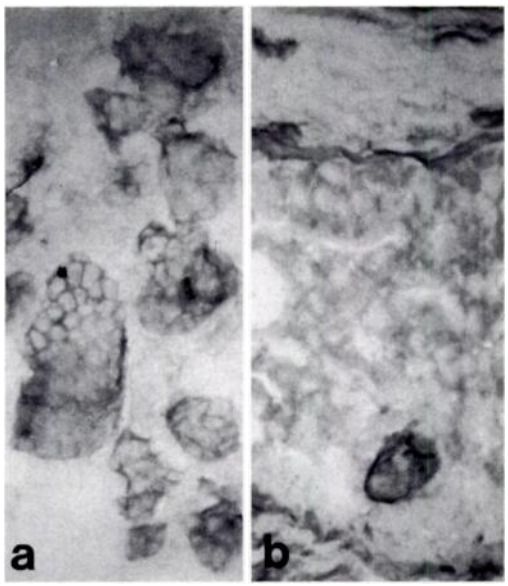

Fig. 1 Immunocytochemical analysis of frozen sections of breast carcinoma with MAb against β4 (*a*) or polyclonal serum against laminin (*b*).

the α6 integrin chain (12); and 3E1, directed against the β4 integrin chain (Telios, San Diego, CA). Rabbit serum directed against human placental laminin (Telios) was used.

Tumors were considered positive when more than 20% of cells were reactive with the MAb tested.

Statistical Analysis. Differences in frequencies were analyzed by the χ^2 test with Yates correction. The survival curve was calculated from the date of surgery, using death from cancer only (overall survival) and recurrence (disease-free survival) as end points. Survival rates were calculated using the actuarial life table method, considering the subgroups identified by the variables examined, and compared using the log-rank test.

Table 2 Analysis of relapses and deaths according to different pathobiological parameters

Parameter	No. of cases	% of relapse	*P*	% of death	*P*
Node					
+	44	34		16	
−	36	19		0	
			0.14		0.01
Size					
≤2 cm	45	18		4	
>2 cm	24	42		12	
			0.03		0.2
$p165^{HER2}$					
−	46	22		7	
+	30	37		13	
			0.15		0.3
α6					
−	45	24		2	
+	35	34		18	
			0.7		0.02
β4					
−	44	23		2	
+	36	33		17	
			0.3		0.02
Laminin					
−	46	35		4	
+	30	20		17	
			0.2		0.06

Table 3 Analysis of relapses and deaths according to laminin production and α6β4 expression

Laminin production	α6β4 expression	No. of cases	% relapse	% death	*P*
+	+	19	32	26	<0.01
	−	11	0	0	
−	+	16	37	6	NS[a]
	−	30	33	3	

[a] NS, not significant.

RESULTS

Expression of the β4 integrin subunit was detected in 36 of the 80 cases (45%). The expression of β4 integrin subunit was then analyzed in relation to clinical and pathological parameters, such as tumor size, nodal status, degree of tumor differentiation, lymphoid infiltration, and a panel of biological markers associated with tumor progression. β4 expression was found to be strongly associated with α6 integrin subunit expression ($P < 0.01$) and laminin production by tumor cells ($P = 0.01$), whereas, except for a borderline correlation with p53 positivity ($P = 0.06$), β4 expression was not related to any of tumor progression markers tested (Table 1). All cases expressing the α6β4 integrin showed pericellular staining, with a positivity ranging from 50 to 100% of

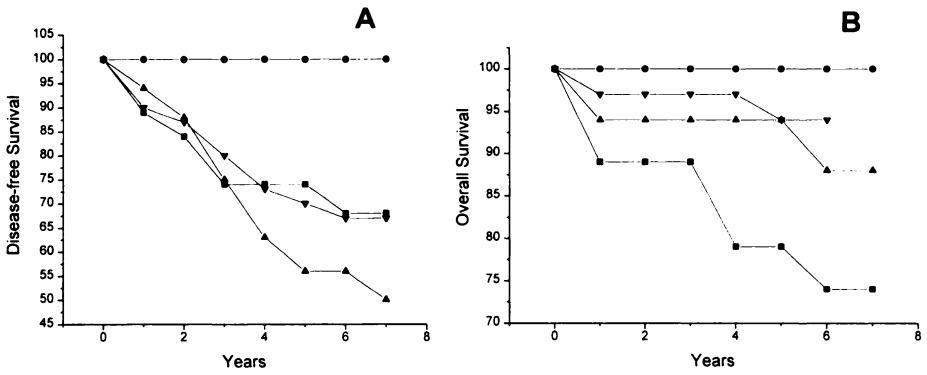

Fig. 2 Disease-free (*A*) and overall (*B*) survival rates of patients with breast carcinomas according to β4 expression and laminin production. ●, α6β4-negative, laminin-positive cases; ■, α6β4-positive, laminin-positive cases; ▼, α6β4-negative, laminin-negative cases; ▲, α6β4-positive, laminin-negative cases. *A*, ● *versus* ■, $P = 0.04$; ● *versus* ▲, $P = 0.03$. *B*, ● *versus* ■, $P = 0.07$.

tumor cells (Fig. 1*a*), whereas tumors considered laminin positive showed specific staining at the membrane and/or the cytoplasmic level on the majority of tumor cells (Fig. 1*b*).

The frequency of relapses and deaths for tumor was then analyzed according to pathological and biological parameters. The median follow-up of these cases was 7 years. Significant correlations between relapses and tumor size ($P = 0.03$) and between deaths and nodal status ($P = 0.01$) were found. The expressions of both α6 and β4 were significantly associated with death ($P = 0.02$), whereas laminin production was only borderline significant ($P = 0.06$; Table 2). Analysis of disease outcome in relation to expression of α6β4 and laminin revealed significant differences in relapse and survival (Table 3). The best outcome was observed for patients with tumors producing laminin but not expressing α6β4 integrin, whereas the worst outcome was found for α6β4-positive, laminin-positive tumors. The other two groups, both negative for laminin production, showed a relapse rate comparable to that of α6β4-positive, laminin-positive tumors, but they showed a lower death frequency (Fig. 2).

Analysis of these four tumor subsets for pathobiological features, including nodal status, tumor size, and other markers, such as $p185^{HER2}$ and p53, revealed no significant differences. Indeed, each subset presented as follows: 80% of cases had tumor sizes of ≤2 cm, grades I–II, and hormone receptor positivity; 50% of cases had lymph node infiltration and $p185^{HER2}$ and bcl-2 expression; and 20% of cases expressed EGFR. p53 positivity was higher in α6β4-positive tumors (20%) than it was in α6β4-negative tumors (10%), independent of laminin production by tumor cells.

DISCUSSION

The present data show that breast carcinomas producing laminin also frequently express the β4 integrin subunit in association with the α6 subunit. The finding that β4 is consistently expressed in association with α6 is in keeping with observations in normal tissues, where coexpressed α6 and β4 always associate together, independent of the presence of β1 subunit (13, 15). This suggests that the frequency of breast carcinomas expressing α6β1 is very low and that this integrin has only marginal relevance in tumor progression. Expression of the α6β4 integrin is maintained mainly in tumors producing laminin, raising the possibility of an autocrine regulatory mechanism in which laminin produced by tumor cells interferes with α6β4 expression and/or redistribution. Because the antiserum used in the present study is directed against human placenta laminin, we actually analyzed the production by tumor cells of all of the different laminin isoforms presented in placenta, including kalinin/laminin 5, which is also recognized by α6β4 (5) and is detected only in invading cells of the tumors (27, 28). However, the antilaminin staining observed in our series was distributed throughout the tumor areas with no apparent correlation to invasive malignant cells. Preliminary data indicate that only the tumors stained with the antilaminin polyclonal antibody were also positive with an antilaminin 5 MAb, but the staining was confined to a very small proportion of tumor cells. These findings suggest a coordinated regulation of laminin isoform production in breast carcinomas.

α6β4 expression in breast tumor cells, unlike that in ovary carcinomas (12), was found to be independent of tumor grade or other prognostic factors but was nevertheless significantly associated with the worst patient outcome ($P = 0.02$). This is in keeping with the finding that restoration of α6β4 receptor expression in a β4-deficient rectal carcinoma cell line facilitates its ability to invade both matrigel and collagen I matrices (29). Moreover, high-level expression of α6β4 on mammary carcinoma cells was found to contribute to liver metastasis formation by mediating adhesion of the cancer cells to hepatocytes (30). Interestingly, 4 of the 5 liver metastases observed thus far in our series derived from α6β4-positive primary tumors. However, our findings point to the crucial role of the α6β4 integrin in tumor progression only when it can interact with its appropriate substrate, supporting the hypothesis that laminin(s) also plays an active role in tumor progression. Indeed, although the patients with laminin-positive, β4-positive tumors showed a recurrence rate similar to that of patients with laminin-negative tumors, they clearly had a higher death frequency (80% of relapse patients) than did the laminin-negative group (12% of relapse patients). The absence of significant differences between the different group of tumor patients in overall survival are most likely due to the low number of events thus far. It has been hypothesized that ligand-bound and unbound α6β4 integrins activate distinct intracellular pathways (31). Indeed, laminin-induced binding of the α6β4 receptor was found to induce tyrosine phosphorylation of the β4 subunit followed by recruitment of Shc/Grb2, thus linking the integrin activation to the ras pathway of proliferation. The lack of aggressiveness by laminin-positive, α6β4-neg-

ative tumors may reflect the binding of the adhesion molecule to other laminin receptors that mediate adhesion rather than proliferation and/or migration. The slight increase in relapses seen in patients with laminin-negative, α6β4-positive tumors suggests that α6β4 integrin in these cases interacts with laminin produced by the host, including these patients in the category of the laminin-positive, α6β4-positive cases.

Consistent with previous analyses indicating a correlation between α6 expression and reduced survival (32), our data emphasize the importance of the interaction of laminin with α6β4 rather than α6β4 expression *per se* in influencing breast carcinoma progression. Specific up- and down-regulation of the expression of laminins and integrins by differentiating agents and cytokines, respectively, may help in modulating tumor aggressiveness.

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