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The Tumor Microenvironment: Possible Role of Integrins and the Extracellular Matrix in Tumor Biological Behavior of Intratubular Germ Cell Neoplasia and Testicular Seminomas

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In the present study, we examined the distribution of integrin subunits and extracellular matrix proteins in normal testis, intratubular germ cell neoplasia (ITGCN), and primary and metastatic seminomas. Compared to normal testis in ITGCN, Sertoli cells showed increased expression of α3, α6, and β1 integrin subunits. Malignant intratubular germ cells stained for α3, α6, and β1 integrin subunits. Progression of ITGCN to invasive seminoma was associated with loss of α3 integrin subunit expression by tumor cells. Consequent to this loss, it can be speculated that the strong expression on ITGCN may be related to the noninvasive character of the lesion as is also known from other noninvasive tumors. All tumors showed a strong expression of α6 and β1 integrin subunits. The α5 integrin subunit was weakly expressed in primary seminomas in all stages. No differences were observed in integrin expression between primary and metastatic tumors. The distribution of extracellular matrix proteins was heterogeneous and revealed clear architectural differences between seminomas that may reflect different stages of tumor stroma formation. To our knowledge, the results presented in this study provide the first information on the possible role of tumor-extracellular matrix interactions in the biological behavior of ITGCN and testicular seminomas. (Am J Pathol 1994, 144:1035–1044)

Integrins are integral membrane proteins, involved in cell-cell and cell-matrix interactions, that play a crucial role in numerous physiological and pathological processes including embryogenesis, wound healing, and biological behavior of malignant tumors. They consist of related noncovalently linked α and β chain heterodimers. The extracellular domain of integrins functions as the ligand binding site and both α and β subunits contribute to ligand binding.[1–4] The cytoplasmatic domain is linked to the cytoskeleton[5] and is supposed to generate signals essential for cell function and tissue processes .[1] Receptor avidity and activity are supposed to be under control of other cell-membrane associated molecules,[6] and conversely integrins may function as co-receptors in certain cellular processes.[1] The specificity of ligand binding is cell type dependent.[7]

The classification of integrins is complex, (see Table 1) caused by association of several α subunits with one particular β subunit, association of one α subunit with more than one β subunit, and alternative splicing of α and β subunits.[1,8,9] Further, individual integrins can often bind to more than one ligand, and individual ligands are recognized by more than one integrin.[1–4]

The conformation-dependent recognition site for several of the integrins is the amino-acid sequence arginine-glycine-aspartic acid present in many, but not all, extracellular matrix (ECM) proteins. Other integrins recognize different sequences in ECM proteins or bind to cellular membrane proteins involved in cell-cell interactions.[10,11]

The ECM constitutes basement membranes (BMs) and the interstitial matrix. It is responsible for tissue integrity, and by interaction with integrin and nonintegrin receptors, ECM proteins can direct gene

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expression[12–14] and modulate proliferation, differentiation, and migration of cells.[15]

Malignant tumors are characterized by abnormal proliferation and differentiation.[16] Moreover, tissue integrity is lost by protease-activity of tumor and/or host cells, making it possible for tumor cells to invade BMs and the surrounding interstitial matrix.[17–20] Compared to normal tissues, this invasion is, at least partially, due to abnormal function and expression of integrins on tumor cells[21–26] and qualitative and quantitative changes of the surrounding ECM.[27,28] However, it must be emphasized that tumor cell invasion and development of metastasis is highly regulated and that the mechanisms used by tumor cells to break down mechanical barriers and migrate are analogous to the ones used by normal cells during physiological processes.[17,20]

Intratubular germ cell neoplasia (ITGCN) of the testis represent the precursor lesion of nearly all adult testicular germ cell tumors and is found adjacent to these tumors in a high frequency.[29–31] In untreated patients, ITGCN has been demonstrated to evolve in an invasive tumor, eg, seminoma (SE) and/or nonseminoma.[30]

Until now, little attention has been paid to the role of tumor-host interactions in invasion and metastasis of testicular SE. Therefore, we have analyzed the expression of integrin subunits and their ligands in the different stages of the metastatic cascade of testicular SE to determine whether the expression of integrin subunits and distribution of ECM proteins was correlated to invasion and/or development of metastases.

Materials and Methods

Tissue

Ten histological normal testes, 10 testes containing placental alkaline phosphatase (PLAP) positive intratubular germ cell neoplasia, 13 primary testicular SEs presenting in stage I (tumor confined to the testis), 12 primary testicular SEs presenting in stage II (tumor with regional lymphatic metastasis), one stage III primary testicular SE (tumor with mediastinal and/or supraclavicular metastases), and eight metastases of primary testicular SE were included in this study. Frozen tissue was randomly selected from our files from cases in which sufficient tissue was available.

Antibodies

Rabbit antiserum directed against PLAP (catalog #A268) was obtained from Dako (Glostrup, Denmark). Goat antisera directed against collagen type I (catalog #1310-01) and collagen type IV (catalog #1340-01) were purchased from Southern Biotechnology Associates (Birmingham, UK). Rabbit antisera directed against laminin (catalog #A105), fibronectin (catalog #A101), and vitronectin (catalog #A104) and monoclonal antibodies directed against fibronectin (catalog #A002, clone II) were obtained from Telios (San Diego, CA). Details of the antibodies directed against the integrin subunits are given in Table 2. Peroxidase-conjugated second and third step antisera were obtained from Dako.

Immunohistochemistry

Immunohistochemistry was carried out on 4-μ frozen tissue sections fixed in acetone for 10 minutes at room temperature. The sections were washed for 5 minutes in phosphate-buffered saline after each incubation step. The sections were incubated with optimal dilutions of the antibodies, as determined previously. To decrease nonspecific binding, the final second and third step antibody dilutions contained 1% normal human AB serum. The localization of peroxidase label was visualized using 3-amino-9-ethylcarbazol together with H_2O_2 as a reagent, giving a reddish brown

Table 1. *The VLA Integrin Family*

Receptor	CD	Other names	Mr	Ligands	Main cellular distribution
α1β1	CD49a/CD29	VLA-1	210,130	Coll(I,IV), Ln	F,M,BM, activated T and B-ly, Mus
α2β1	CD49b/CD29	VLA-2	170,130	Coll(I-III,IV)Ln	Pl,F,EN,EP, activated T-Ly
α3β1	CD49c/CD29	VLA-3	130/25,130	Coll(1),Ln,Fn,Epil	Ep,F,BM,B-ly
α4β1	CD49d/CD29	VLA-4,LPAM2	150,130	Fn,VCAM-1,ICAM-2	M,Eo,Ly,F,NC,NK,Th
α5β1	CD49e/CD29	VLA-5	135/25,130	Fn, Inv	Th,T-Ly,F,EP,EN,Pl,PMN,M,Mus
α6β1	CD49f/CD29	VLA-6	120/30,130	Ln, Inv	Pl,T-ly,EP,Th,M
α6β4	CD49f/–	Tsp180		Ln	EP

VLA: very late activation antigen; CD: cluster of differentiation; Coll: collagen; Epil: Epiligrin; Fn: fibronectin; ICAM: intercellular adhesion molecule; Inv: invasin; Ln: laminin; LPAM: lymphocyte Peyer's patch specific adhesion molecule; Tsp: trombospondin; VCAM: vascular cell adhesion molecule; BM: basement membrane associated; EN: endothelial cells; Eo: eosinophils; EP: epithelial cells; F: fibroblasts; Ly: lymphocytes; M: monocytes/macrophages; Mus: muscle; NC: neural crest cells, melanocytes; NK: natural killer cells; Pl: platelets; PMN: polymorphonuclear cell (neutrophil); Th: thymocytes.

Table 2. *Anti-integrin Antibodies Used*

Antibody; clone; catalog #	Subunit	CD	Source/references
TS2/16	β1	29	Springer[64]
A-1A5	β1	29	Hemler[65]
3E1; A054	β4		Telios, San Diego, USA[66]
TS2/7	α1	49a	Hemler[64]
CLB Tromb/4	α2	49b	Sonnenberg[67]
J143	α3	49c	Old[68]
P1B5; A043	α3	49c	Telios, San Diego, USA[69]
B5G10	α4	49d	Hemler[70]
HP2/1	α4	49d	Sanchez-Madrid[71]
BIIG2	α5	49e	Damsky[72]
P1D6; A045	α5	49e	Telios, San Diego, USA[69]
G0H3	α6	49f	Sonnenberg[73]

CD: cluster of differentiation

precipitate. The slides were counterstained with hematoxylin and mounted with Kaisers glycerol-gelatin (Merck, Darmstadt, Germany). Controls included sections on which the application of the primary antibody was omitted or replaced by nonrelevant antibodies.

Results

Expression of Integrin Subunits in Normal Testis, ITGCN, Primary SE and Metastases of SE

Normal testes, ITGCN, primary SE and metastases of SE showed a variable immunohistochemical expression of integrin subunits, summarized in Tables 3 and 4. The α3 subunit was expressed by Sertoli cells and a subset of intratubular germ cells. A remarkable feature was the very localized strong α6 integrin subunit expression at the basal side of intratubular cells (Figure 1A). In atrophic tubules and ITGCN containing tubules, the expression of both subunits on Sertoli cells was increased (Figure 1A). The distribution of the β1 subunit was concordant with the distribution of the α integrin subunits. PLAP-positive malignant intratubular germ cells showed a strong nonpolarized expression of the α3 and α6 integrin subunits (Figure 1, B and C). The β1 integrin subunit was weakly expressed. In primary SEs, the α3 (three of 25) and α5 (11 of 25) integrin subunits were weakly expressed in all stages. All tumors showed a strong expression of α6 (Figure 2A) and β1 integrin subunits. No significant differences were observed in the integrin expression between primary and metastatic lesions. The expression of the α5 integrin subunit on metastatic SE cells is shown in Figure 2B. In one metastasis, seminoma cells showed expression of the α6, β1, and β4 integrin subunits with increased density at the tumor-stroma border (Figure 2, C and D).

Composition of the ECM in Normal Testes, ITGCN, Primary SE, and Metastases of SE

The lamina propria and the tubular BM were composed of laminin, collagen type IV, collagen type I, fibronectin, and vitronectin. In atrophic and ITGCN-containing tubules, the lamina propria and tubular BM were thickened and the BM invaginated into the tubular lumen. In some tubules, staining for ECM proteins revealed double tracking of the tubular wall (Figure 3A). Although the BM was generally thickened, local interruptions of the testicular BM were also observed (Figure 3A). Weak staining for vitronectin was seen in some tubules containing ITGCN and intratubular SE. In one case, tubular structures composed of laminin, collagen type IV, collagen type I, fibronectin, and vitronectin were seen in tumor nodules adjacent to preexistent tubules (Figure 3B).

Based on the distribution of collagen type I, three types of stromal reaction could be recognized (Figure 4, A, B, and C). In two tumors, the stroma was composed of relatively small fibrovascular septa, which showed a regular distribution in the tumors (stromal reaction type I). Large fibrous bands giving the tumor tissue a nodular appearance were seen in 11 tumors (stromal reaction type III). The other 13 tumors showed a stromal reaction of the intermediate type

Table 3. *Integrin Subunit Distribution in Normal Testes and Testes with Intratubular Germ Cell Neoplasia*

Integrin subunit	α1	α2	α3	α4	α5	α6	β1	β4
Vascular system								
Endothelium	+/−	±	+	±	+	+	+	+
Smooth muscle	+	−	+	−	−	−	+	−
Leydig cells	±/−	−	±/−	−	−	±/−	±	−
Lamina propria	±	−	±	−	±	±	+	−
Sertoli cells	−	−	±/+*	−	−	±/+*‡	±	−
Spermatogenesis	−	−	±†	−	−	+‡	+	−
ITGCN§	−	−	+	−	−	+	+	±/−

Expression of integrin subunits: − negative, ±/− equivocal, ± weak, + positive. * Increased expression of the α3 integrin subunit on Sertoli cells in atrophic and ITGCN containing testis. † α3 integrin subunit expression on a subset of intratubular germ cells. ‡ Localized α6 integrin subunit expression at the basal side of intratubular cells. § Intratubular germ cell neoplasia.

Table 4. *Expression of Integrin Subunits in Primary Testicular Seminomas and Metastases of Primary Testicular Seminomas*

Integrin subunit	α1	α2	α3	α4	α5	α6	β1	β4
SE stage I	–	–	±2/12	–	±4/12	+12/12	+12/12	±/–4/13
SE stage II/III	–	–	±1/13	–	±7/13	+12/13	+12/13	±/–2/13
Metastases	–	–	+/±2/8	–	±4/8	+8/8	+8/8	±1/8

Expression of integrin subunits: – negative, ±/– equivocal, ± weak, + positive.

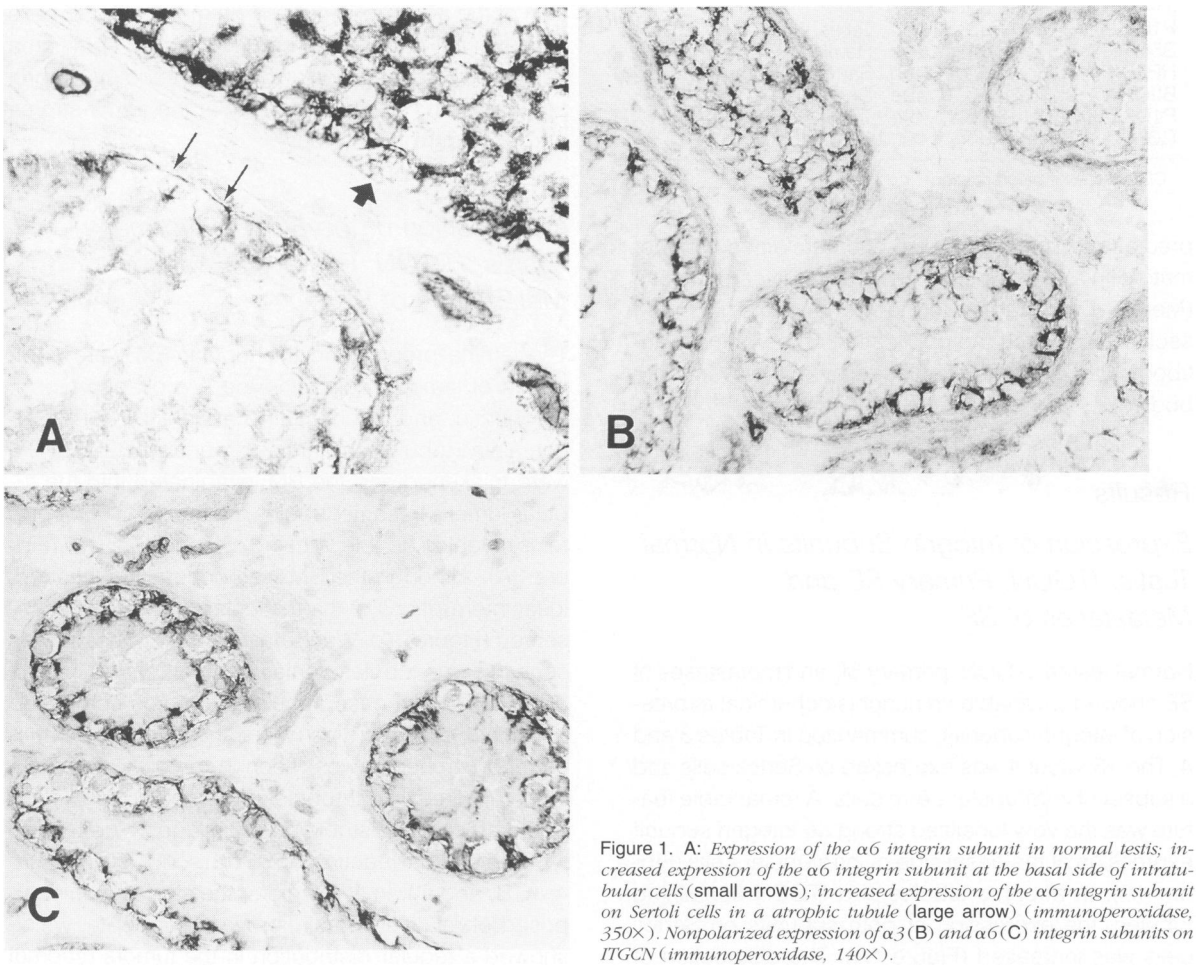

Figure 1. A: *Expression of the α6 integrin subunit in normal testis; increased expression of the α6 integrin subunit at the basal side of intratubular cells* (small arrows); *increased expression of the α6 integrin subunit on Sertoli cells in a atrophic tubule* (large arrow) (*immunoperoxidase, 350×*). *Nonpolarized expression of α3* (B) *and α6* (C) *integrin subunits on ITGCN* (*immunoperoxidase, 140×*).

with small fibrovascular septa as well as large fibrous bands (stromal reaction type II). The differences between the three types of stromal reaction were relative but reproducible, as typing of the stromal staining pattern by two independent observers (including repeated typing with time interval) gave almost identical results.

The fibrovascular septa showed a diffuse weak staining of laminin and collagen type IV, and strong staining of collagen type I, fibronectin and vitronectin. Small band-like structures resembling BMs, composed of laminin, collagen type I, collagen type IV, fibronectin, and vitronectin, were randomly distributed in all primary SEs. In addition to these structures, in seven SEs, a linear BM was present at the interface of tumor cells and stroma (Figure 5B). Codistribution of ECM proteins was not always evident (Figure 5, c and d).

In metastases of SE, the composition of the ECM was similar to that in primary SE. All but one metastases showed a type III stromal reaction. In one metastasis, tumor cells were separated from the stroma by a linear BM, whereas in the other seven metastases, the distribution of BMs was similar to that found in primary SE.

Discussion

Invasive growth and the development of metastasis is dependent on a repeated sequence of events, first

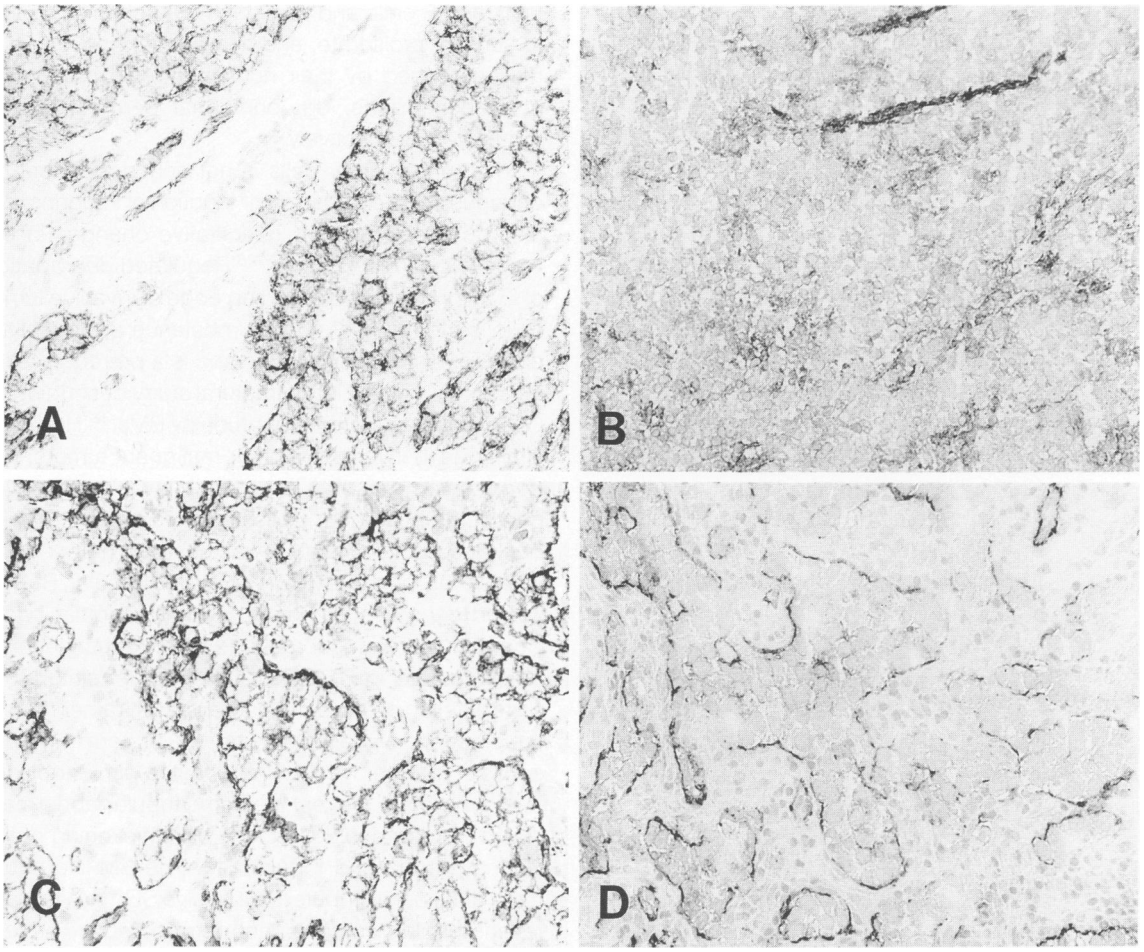

Figure 2. *Nonpolarized expression of the α6 integrin subunit on SE cells in a primary SE* (**A**). *Weak expression of the α5 integrin subunit on SE cells in a metastasis of a primary SE* (**B**). *At the tumor-stroma border α6* (**C**) *and β4* (**D**) *integrin subunits show increased expression on SE cells in a metastasis of a primary SE* (*immunoperoxidase, 140×*).

described by Liotta as the three-step hypothesis of invasion.[17–19] The first step comprises the adhesion of tumor cells to BMs, mediated by integrin and non-integrin receptors. This adhesion elicits proteinase-mediated degradation of BM components like laminin and collagen type IV at the leading edge of tumor cells. Locomotion of tumor cells through the localized zone of BM-lysis is the final step. This multistep process is highly regulated, and the outcome in invasive tumors is the result of an imbalance between positive and negative regulatory factors, which are operative in physiological processes as well.[17,20]

It is evident that the interaction of tumor cells with ECM proteins, mediated in part via integrins, plays a crucial role in biological behavior of malignant tumors. Experimental studies with α5β1 integrin receptor-deficient or overexpressing variants of Chinese hamster ovary cells and human rhabdomyosarcoma cells transfected with DNA coding for the α2 integrin subunit suggest that changes in integrin expression explain, at least in part, some of the characteristics of transformed cells, including their migratory, invasive, and metastatic behavior.[23–26]

Although not consistently and dependent on the cell type, malignant cells often express an altered pattern of integrins compared with their nontumorigenic benign counterparts.[33–35] Furthermore (in contrast to an often localized pattern on the membrane of normal cells), in malignancy, integrins are diffusely distributed over the cell surface,[23,36] and their function is supposed to be changed.[24,37] The expression of integrin subunits on intratubular germ cells, malignant intratubular germ cells, and invasive SE is concordant with these observations. Progression of ITGCN to invasive SE is associated with loss of α3 integrin subunit expression. Because invasive SE cells show no or weak expression of the α3 integrin subunit, the strong expression on ITGCN may be related to the nonin-

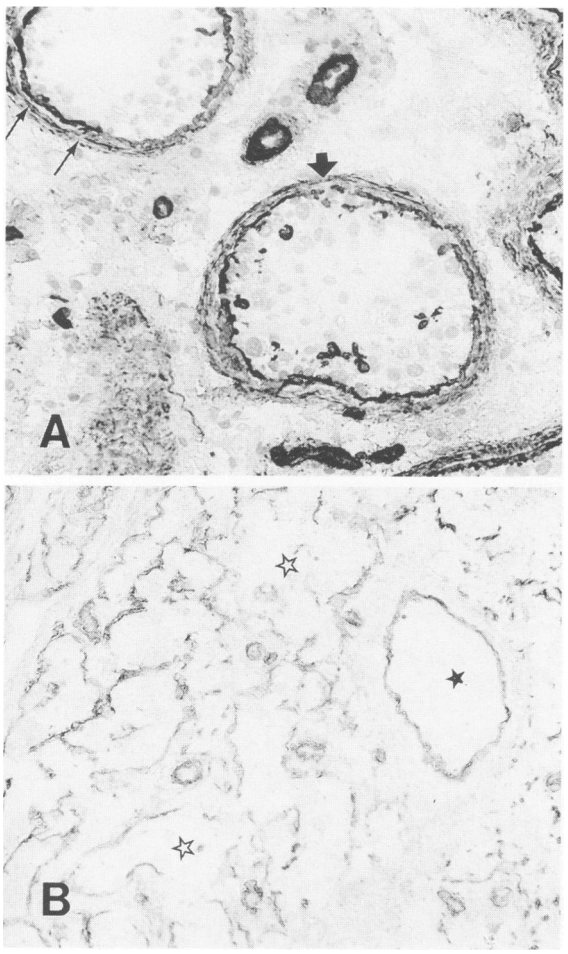

Figure 3. *Distribution of laminin in ITGCN-containing testis with double tracking of the tubular wall* (small arrows). *The BM is irregular with invaginations into the tubular lumen. Interruptions of the BM are locally present* (large arrow) A: *Tubular structures stained for laminin in tumor nodules* (open stars) *adjacent to a preexistent tubule* (black star) (B) (*immunoperoxidase, 140×*).

vasive character of the lesion. Similarly, a high expression of $\alpha 2$ and $\alpha 3$ integrin subunits has been suggested to account for the indolent nature of basal cell carcinoma.[38]

The $\alpha 5\beta 1$ and $\alpha 6\beta 1$ integrin receptors function as receptors for fibronectin[39,40] and laminin,[41] respectively. The nonpolarized expression of these integrin subunits in invasive primary and metastatic SE is compatible with previous studies, suggesting a prominent role for adhesion of tumor cells to fibronectin and laminin in invasion and metastasis.[19,23–25,42–44] Whereas the $\alpha 6\beta 1$ integrin receptor is implicated in promoting invasion of tumor cells,[43] transfection studies with Chinese hamster ovary cells rather point to a suppressor role of the $\alpha 5\beta 1$ integrin receptor in invasion and metastasis.[24,45] Future studies, especially those with SE cell lines, will have to elucidate the function of $\alpha 5$, $\alpha 6$, and $\beta 1$ integrin subunits on SE cells and reveal if the mechanisms used to survive, proliferate, and migrate are analogous to the ones used by their nontumorigenic precursors and counterparts, eg, primordial germ cells and gonocytes, respectively.

Invasion of tumor cells is not only dependent on changes in the expression or function of integrins, but also on qualitative and quantitative changes in the composition of the ECM.[27,28] Regulated degradation of ECM proteins at the leading edge of invasive tumor cells is the result of a local imbalance of proteolytic enzymes and their inhibitors and is a prerequisite for invasion.[17,20] In an ultrastructural study describing ITGCN, Schulze found intact tubular BMs.[46] However, similar as in invasion of other malignant tumors, BM degradation probably is a step in invasion of malignant intratubular germ cells, as microinvasive SE with destruction of the tubular wall has been reported,[32] whereas in our study in ITGCN, the tubular BM revealed gaps besides thickened and irregular parts. Changes involving increased ECM production in tubules containing ITGCN may in fact be related to a disregulation of testicular homeostasis, rather than to the presence of malignant intratubular germ cells per se, as cryptorchid testes[47] and testes with Sertoli cell only syndrome[48] reveal the same tubular abnormalities, including double tracking and thickening of the lamina propria and tubular BM. The BM-like structures diffusely distributed in invasive SE may well represent tubular remnants, not degradated by proteolytic enzymes. However, as Schulze suggested, BM-like structures in invasive SE may as well be newly deposited,[46] as illustrated by the linear distribution of BM proteins at the interface of SE cells and stroma. This deposition is ineffective because BMs in malignant tumors, including testicular SE, often reveal (ultrastructural) abnormalities.[49–52] Whatever the origin of these structures and analogous to the adhesion of Sertoli cells and spermatogenic cells to the tubular BM,[53] adhesion of SE cells to BM structures and/or the interstitial matrix may hamper their migration and bear on the development of metastasis.

It is supposed that after tumor cells have invaded the surrounding tissue, they elicit the formation of a primitive stroma. This stroma consists of plasma-derived cross-linked fibrin and fibronectin.[54–56] Like fibronectin, vitronectin is found in the circulation[57] and might be trapped in the fibrin-fibronectin gel as well. The fibrin-fibronectin gel serves as a provisional matrix that facilitates and regulates influx of endothelial cells, fibroblasts, and other host cells.[56,58] Subsequently, by deposition of tumor or host cell-derived collagen type III and I, the provisional matrix is replaced by mature stroma.[54–56]

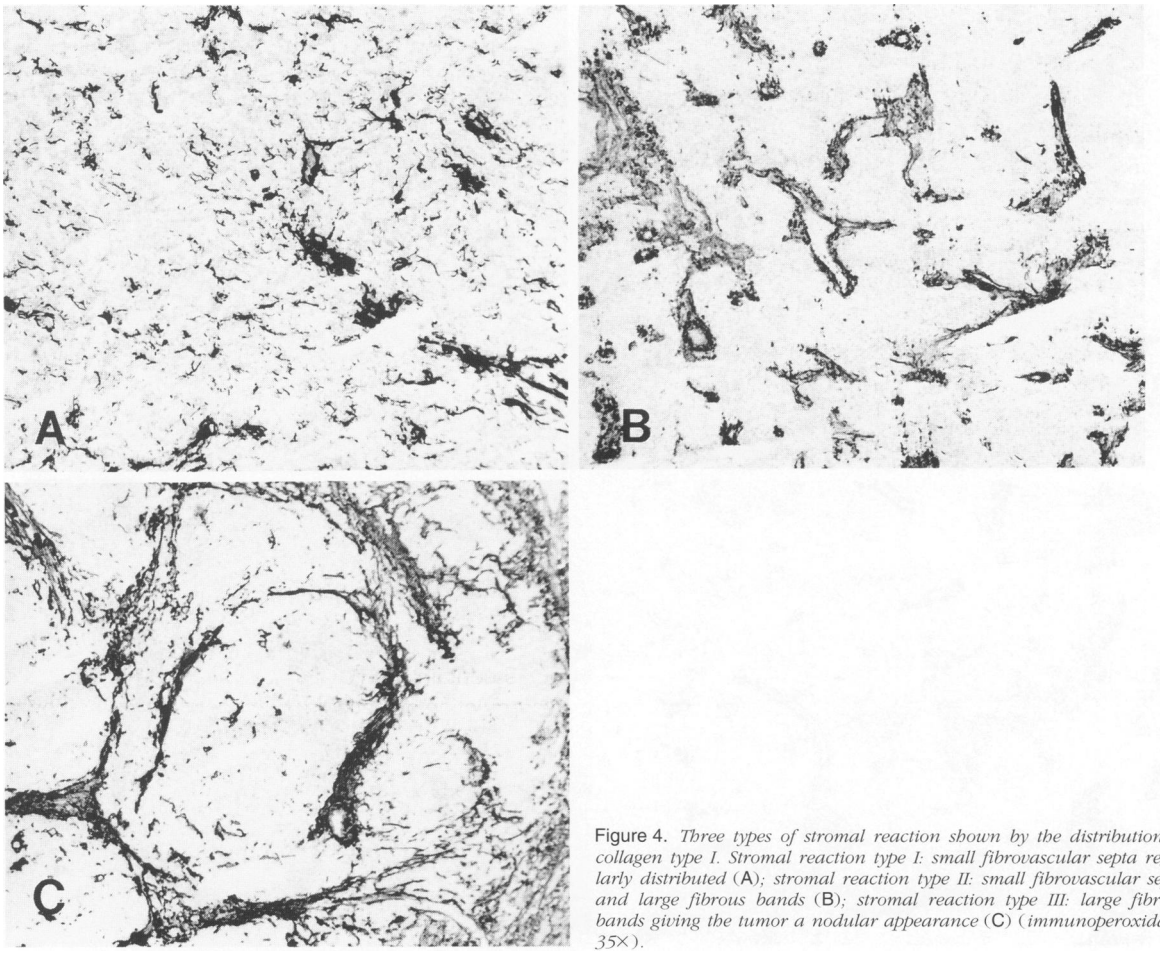

Figure 4. *Three types of stromal reaction shown by the distribution of collagen type I. Stromal reaction type I: small fibrovascular septa regularly distributed* (A)*; stromal reaction type II: small fibrovascular septa and large fibrous bands* (B)*; stromal reaction type III: large fibrous bands giving the tumor a nodular appearance* (C) *(immunoperoxidase, 35×).*

In SE, the mature stroma, composed of collagen type I, fibronectin, and vitronectin, was differentially distributed between different tumors; three types of stromal reaction could be recognized. Because differences between the three types of stromal reaction were partly relative, they may represent different stages in the ongoing process of fibrovascular stroma formation. In this context, the tumors with a nodular appearance (type III stromal reaction) are supposed to represent the more progressed seminomas.[59]

It is evident that the stroma functions as a mechanical scaffold that defines tissue boundaries. However, by modulating cell function, the stroma may have also an active role in numerous physiological and pathological processes.[51,60] As constituents of the stroma, fibroblasts,[61] ECM proteins,[62] and lymphocytes[63] have been reported to modulate tumor cell function, including proliferation. The coincident finding of low proliferative activity and fibrotic tissue in SE is in line with this observation.[59]

The results presented in this study provide information on the possible role of tumor-ECM interactions in the biological behavior of ITGCN and testicular SE. More specifically, our results demonstrate that SE are heterogeneous with respect to integrin subunit expression and composition of the ECM. Moreover, the dynamic interplay between tumor cells and their surrounding interstitial matrix is illustrated. It is evident that further studies will have to be undertaken to determine the significance of our observations with respect to tumor growth, invasion, and metastasis.

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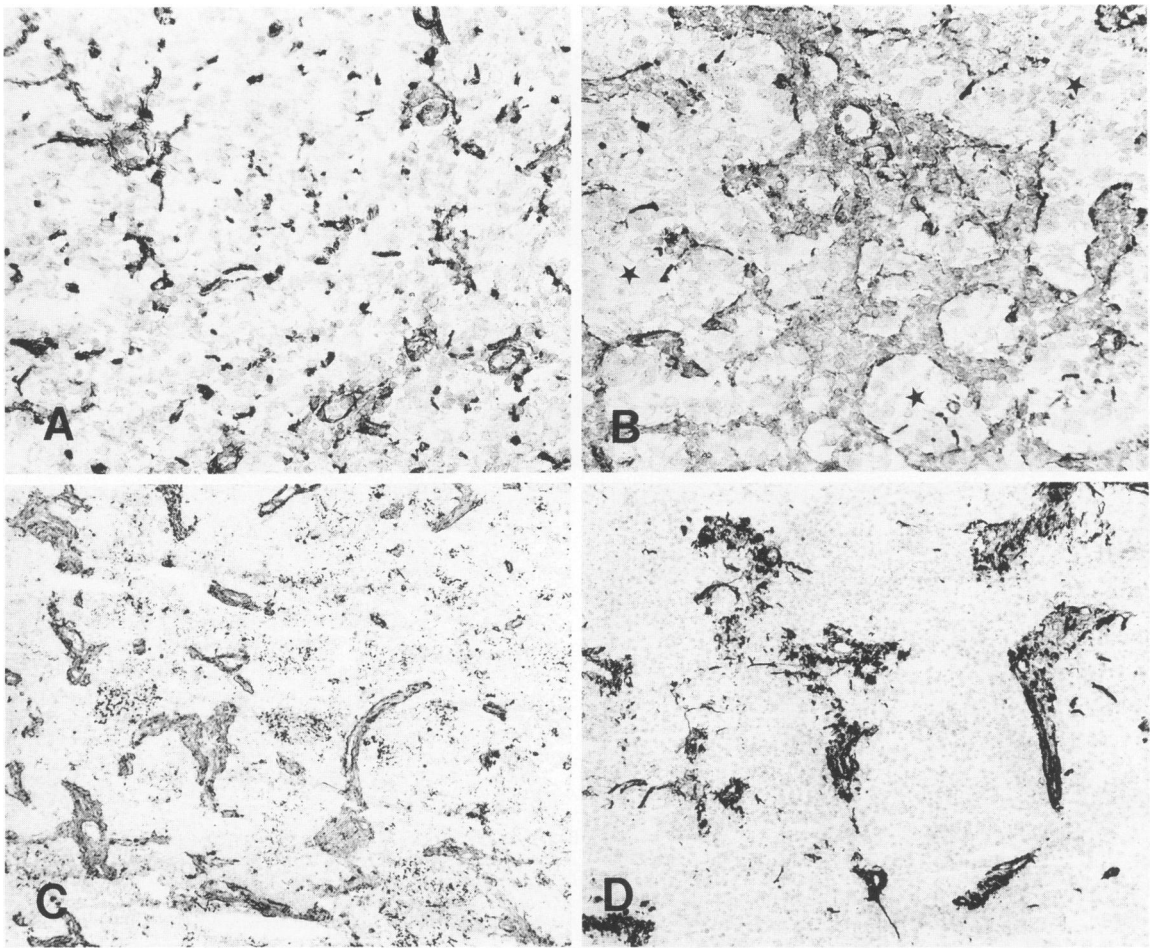

Figure 5. A: *Randomly distributed small band-like structures stained for collagen type IV in a primary SE.* B: *Fibrovascular septa and SE cells (indicated by* stars*) separated by a linear BM as revealed by staining for collagen type IV in a primary SE* (B) (*immunoperoxidase, 140×*). *The distribution of vitronectin* (C) *is more extensive than collagen type I in the same primary tumor* (D) (*immunoperoxidase, 56×*).

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