Correlation between integrin α5 expression and the malignant phenotype of transitional cell carcinoma

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Summary We examined the expression of α1, α2, α3, α4, α5 and β1 integrin on 36 transitional cell cancers (TCCs) in the bladder by immunohistochemistry. Only α2, α3 and β1 were detected on normal transitional cell epithelium, but four TCCs (12.5%) revealed positive staining for α1, seven (19.4%) for α4 and seven (20%) for α5. These altered expressions of integrin α chain were more frequent in histologically higher stage or grade of TCC, and a correlation was found between increased α5 expression and histological stage. α5 was positive in 6 (35.3%) of 17 invasive TCCs whereas only 1 (5.9%) of 17 superficial TCCs. Flow cytometric analysis on bladder cancer cell lines showed that T24 and HT1376, which are undifferentiated TCC cell lines, highly expressed α5 and β1. Also, SCaBER, which is derived from urinary bladder squamous cell cancer and which is recognised as the most malignant phenotype after metaplasia of transitional epithelium, had α5 and β1. However, RT4, which is derived from transitional cell papilloma, showed no expression of α5. Furthermore, reverse transcriptase–polymerase chain reaction (RT–PCR) showed the presence of mRNA of α5 on T24, SCaBER and HT1376, but not on RT4. Taken together, it seems that the presence of α5 integrin might be a more malignant phenotype in transitional cell carcinoma.

Keywords: integrin; VLA-5; transitional cell cancer; immunohistochemistry; flow cytometry; reverse transcriptase–polymerase chain reaction

In the process of tumour invasion and metastasis, the interaction between tumour cells and extracellular matrix (ECM), such as laminin, fibronectin and collagens, has a crucial role (Nicolson and Winkelmake, 1975; Horn and Tang, 1992). This interaction is facilitated through adhesion receptors such as integrins. Integrins are a family of cell-surface proteins that mediate cell adhesion to ECM and signal transmission to the cell interior (Hynes, 1990; Albeda, 1993). They are composed of two subunits, α and β, each of which spans the plasma membrane. Distinct α-subunits combine with common or related β-subunits to form functionally distinct receptors.

Integrin distributions have been studied in a number of tissues, including malignant tumours. Recent studies showed that patterns of integrin expression on tumour cells were different compared with normal counterparts and suggested that altered integrin expression may contribute to the invasive or metastatic phenotype. For example, the loss of integrin expression was reported in epithelial neoplasms, such as carcinomas of the breast, colon, pancreas and skin (Pignatelli et al., 1990; Zutter et al., 1990; Hall et al., 1991; Stamp and Pignatelli, 1991). On the other hand, up-regulation of αVβ3 was described in malignant melanoma and glioblastoma multiforme, and acquisition of α4β1 has been described in malignant melanoma and renal cell carcinoma (Cheresh et al., 1989; Gladson and Cheresh, 1991; Tomita et al., 1995). In transitional cell carcinoma (TCC), progressive loss of α2 integrin expression from normal urothelial cells through invasive cancers was reported and some α5 integrin was expressed on high stage TCC (Leibert et al., 1994).

VLA-5 (α5β1 integrin) is a fibronectin receptor whose expression is often reduced in tumour cells (Plantefaber and Hynes, 1989). In addition, increasing the expression of the α5β1 integrin by gene transfer decreases the formation of tumours on Chinese hamster ovary cells, suggesting the presence of VLA-5 on tumour cells might be a disadvantage for their proliferation (Giancotti and Ruoslahti, 1990). Indeed, after transfection, they showed less migratory, reacquired features of normal growth control in culture, resulting in lost ability to form tumours when injected subcutaneously into nude mice (Giancotti and Ruoslahti, 1990). Several other studies, including some on human tumour cells, confirmed the correlation between low VLA-5 expression and malignant transformation or higher malignant potential (Varnet et al., 1992; Witkowski et al., 1993).

The expression of VLA-5 on high stage TCC in a previous report seemed curious. Therefore, we focused our interest on the expression of VLA-5 on TCC and the normal transitional cell and its mRNA. We examined the expression of integrins on 36 TCCs and six normal transitional cells by immunohistochemistry. Flow cytometric analysis and RT–PCR for VLA-5 or mRNA of α5 integrin on bladder cancer cell lines and normal transitional cell line were also performed. We showed that the presence of VLA-5 might indicate a more malignant phenotype, as for TCC.

Materials and methods

Tissue specimens

Tumour specimens were obtained from 36 patients (25 males and 11 females) who had undergone total cystectomy or transurethral resection for bladder cancer. The mean age at the time of operation was 67.9 years, ranging from 41 to 84 years. Six specimens of normal urinary bladder epithelium were collected from a histologically unaffected portion of the bladder. Tissue samples were embedded in an optimum cold temperature compound (Miles Laboratories, Naperville, IL, USA) and were quickly frozen in isopentane, precooled in dry ice aceton. These blocks were stored at –80°C until 5 μm serial sections were cut using a cryostat. Histological examination was performed on haematoxylin and eosin-stained tissue sections. Tumours were graded and staged according to the criteria of the World Health Organization.

Reagents

Monoclonal antibodies (Mabs) used in this study were as follows: TS2/7 against α1, P1E6 against α2, P1B5 against α3, P4G9 against α4, P1D6 against α5, 4B4 against β1. Polyclonal anti-α5 antibody AB1928 was also used. These Mabs were purchased from Telios Pharmaceuticals (San Diego, CA, USA), except for 4B4 which was purchased from Corter (Hialeah, FL, USA) and AB1928, which was

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purchased from Chemicon International (Temecula, CA, USA). Optimal dilution for each antibody was determined by staining specimens of lymph nodes obtained during nephrectomy for renal cell carcinoma without tumour metastasis.

Immunoperoxidase staining

Immunoperoxidase staining was performed using the streptavidin–biotin bridge technique described previously (Tomita et al., 1990). Briefly, serial sections were air dried and fixed in cold acetone. After rehydration with phosphate-buffered saline (PBS), sections were incubated in PBS containing 20% normal sheep serum (Antibodies, Davis, CA, USA) for 30 min, and endogenous biotin was blocked using an endogenous biotin blocking kit (Vector Laboratories, Burlingame, CA, USA). They were then incubated with mouse MAb for 60 min, followed by incubation with biotinylated sheep anti-mouse immunoglobulin (Amersham International, Amersham, Bucks, UK) in PBS containing 20% human type AB serum (Biological Specialties, Lansdale, PA). Subsequently, they were incubated with streptavidin–peroxidase (Amersham) for 15 min. Each step was followed by washing in PBS with three changes of buffer. Finally, the sections were immersed in 0.05 mol·l⁻¹ Tris-HCl buffer containing 0.05% diaminobenzene and 0.01% hydrogen peroxide for 4–20 min to visualise the reaction products. Specimens were counterstained in Mayer’s haematoxylin and mounted after dehydrating in graded ethanol and xylene. Tumours were considered as positive when positive tumour cells were observed in the specimen, although the tumour tissue showed various staining pattern when reacted with anti-α5 MAb. Statistical analysis was done by using the chi-square test.

Flow cytometric analysis of bladder cancer cell lines

To investigate differences in α5 expression among bladder cancer cell lines with various characteristics, we used four established bladder cancer cell lines and a normal transitional cell line obtained from the American Type Culture Collection (ATCC). Three cancer cell lines, T24 (Bubenik et al., 1973), SCaBER (O’Toole et al., 1976) and HT1376 (Rasheed et al., 1977) were derived from high-grade bladder cancers; one, RT4 (Rigby and Franks, 1970), was derived from papilloma and HT160 was derived from normal fetal bladder cells. These cell lines were cultured in 3055 (Coaster, CA, USA) 25 cm² tissue culture flasks in complete medium (RPMI-1640 medium containing 10% fetal calf serum). For flow cytometric analysis, cells were stained by the indirect immunofluorescence method as described previously (Tomita et al., 1990). Briefly, tumour cell suspensions were prepared by treatment with 0.125% trypsin and 0.02% EDTA. Tumour cells were reacted with anti-α5 or β1 MAb in PBS supplemented with 2% fetal calf serum and 0.02% sodium azide for 30 min at 4°C. After washing twice by centrifugation, cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse Ig (Tago, Burlingame, CA, USA) for 30 min at 4°C. Subsequently, the cells were washed three times and analysed by flow cytometry (FACScan, Becton-Dickinson).

Reverse transcription of RNA followed by the polymerase chain reaction (RT–PCR) analysis

Total RNA was isolated from cell lines using RNAzol (Biotex Laboratories, USA) according to the instructions of the manufacturers. Total RNA (10 μg) was used for cDNA synthesis. First-strand cDNA solution (2 μl) was then used for PCR, with primers designed to amplify a 1421 bp α5 cDNA sequence from bp 1625 to bp 3046 (sense primer sequence: 5'-ACAAGTGTCAGTCCATTG), PCR was performed in 50 μl buffer (50 mmol·l⁻¹ potassium chloride; 10 mmol·l⁻¹ Tris-HCl, pH 8.4; 1.5 mmol·l⁻¹ magnesium chloride and 200 ng·μl⁻¹ gelatin), with 200 mmol·l⁻¹ of each dNTP, 2.5 mmol·l⁻¹ of each oligonucleotide primer, and 2.5 U of Taq DNA polymerase (Boehringer Mannheim, Germany). Thirty-five cycles of 1 min denaturation at 94°C, 1 min annealing at 60°C, and a 1.5 min extension step at 72°C were performed. At the end of the 35 cycles, an additional 10 min extension step at 72°C was added. Subsequently, to confirm the specificity of this product, nested PCR was performed using 5 μl of PCR product with primers designed to amplify a 1268 bp α5 cDNA sequence from bp 1657 to bp 2925 (sense primer sequence: 5'-TCAGGGATCCAACTTCAGCTGGACAGCAGAAGCA, antisense primer sequence: 5'-GATC-GAAAATTCGGCATCTTCAAGGCTTGTATACAAA).

Results

In situ expressions of integrins on TCCs

When normal transitional cells were stained with anti-integrin MAbS, constitutional expressions of α2, α3 and β1 were detected. However, α1, α4 and α5 were negative for all six normal transitional cell specimens. On the contrary, four TCCs (12.5%) revealed positive staining for α1, 7 (19.4%) for α2, 4 (10%) for α3 (Table I). Three TCCs (8.3%) showed decreased expression of α2. These altered expressions of integrin α chain were more frequent in histologically higher stage or higher grade of TCC (Table I). Although statistically significant, a correlation was found between increased α5 expression and the histological stages. α5 was positive in 6 (35.3%) of 17 invasive TCCs but only 1 (5.9%) of 17 superficial TCCs (Table II).

Figure 1 Immunohistochemical staining for α5 integrin. (a) Grade 3 invasive (pT4) bladder TCC positively stained with anti-α5 integrin. (b) Grade 1 non-invasive (pT1) bladder TCC was negative for α5. Scale bars = 75 μm.
Flow cytometric analysis of the expression of α5 and β1 integrins on human TCC cell lines

Results of immunohistochemical staining against α5 prompted us to investigate its expression on bladder cancer cells and the transitional cell line. Flow cytometric analyses showed α5 molecules on T24 and HT1376, which are TCC cell lines with malignant phenotype, and SCaBER, which is a bladder SCC cell line, but not on RT4 derived from benign papilloma. However, HTB160, which is a normal TCC line, showed the presence of α5. All of them showed positive staining against β1 (Figure 2).

Detection of α5 mRNA by RT–PCR

To confirm the presence of α5 mRNA, we performed nested RT–PCR using two sets of primers. Integrin α5 cDNA fragments of the expected size could be amplified from T24, SCaBER, HT1376 and HTB160 mRNA. However, RT–PCR for RT4 mRNA did not show any bands (Figure 3).

Discussion

In order to investigate the alteration of integrin expression of TCC, we immunohistochemically examined α1, α2, α3, α4, α5 and β1 integrin on bladder TCC as well as normal transitional epithelium. When normal transitional cell epithelium was stained with the panel of MAbs, only α2, α3 and β1 were positive. On the other hand, some TCCs expressed α1, α4 and α5, and these altered expressions of integrins were observed relatively frequently in the higher grade and/or stage tumours. These results suggest that increased expression of α1, α4 and α5 might change the character of TCC to ECM and facilitate tumour invasion or metastasis. Also, a significant correlation was found only between increased α5 expression and the histological stage. Leibert et al. (1994) also showed that α5 integrin was expressed on high-stage TCC but not on low-stage tumours. However, α5 was detected on TCC in a rather smaller number of tumours than in the present study, and there was no positive staining of α4. These discrepancies may be explained by the difference in staining methods and/or MAbs used. We might use a possibly more sensitive staining procedure, amplifying positive staining by the streptavidin–biotin system, which we used in other studies (Tomita et al., 1990, 1993).

A member of the integrin β1 subfamily, VLA-5 (α5β1), is a fibronectin receptor, and its expression is often reduced in tumour cells (Plantefaber and Hynes, 1989). In addition, increase in α5β1 expression by gene transfection decreases the formation of tumours on Chinese hamster ovary cells, suggesting that the presence of VLA-5 on cells might be a disadvantage for tumour cell proliferation by transducing growth-inhibitory stimuli from fibronectin (Scherer et al., 1991). Indeed, after transfection, they showed less migration and reacquired the features of normal growth control in culture, resulting in loss of the ability to form tumours when injected subcutaneously into nude mice (Giancotti and Ruoslahti, 1990). Several other studies, including human tumour cells, have confirmed the correlation between low
The expression of α5 and β1 chain was frequent on human TCC cell lines and transitional cell line. Both expressions were examined on T24, HT1376, SCaBER, RT4 and HTB160. RT4 did not express α5 integrin.

VLA-5 expression and malignant transformation or a higher malignancy (Varner et al., 1992; Witkowski et al., 1993).

The present study on TCC, however, showed a more frequent expression of α5 and β1 chain on TCCs of higher grade and histopathological stage. In addition, examination of normal transitional cells showed no α5 staining. Compatible with the results of immunohistochemistry on TCC, flow cytometric analysis on cultured bladder cancer cell lines showed that T24, SCaBER and HT1376, which are undifferentiated TCC or SCC cell lines said to have a higher malignant character, revealed high expression of α5 and β1, whereas RT4, which is derived from transitional cell papilloma and preserves a well-differentiated character, showed no expression of α5. SCC of the urinary bladder is recognised as the most malignant phenotype after metaplasia of transitional epithelium. Furthermore, RT–PCR showed the presence of mRNA on T24, SCaBER and HT1376, but not on RT4. Taken together, it seems more likely that, as for TCC, the presence of VLA-5 might be a more malignant phenotype. There have been some reports of the presence of VLA-5 with malignant phenotype as in the present study (Terpe et al., 1993; Leibert et al., 1994). Interestingly, HTB160, which is a normal TCC line, showed positive staining against α5. However, HTB160 was developed from fetal bladder. It is possible that the expression of integrin is different between adult and fetal bladder because its expression is reported to be different between adult and fetal kidney (Korhonen et al., 1990).

In conclusion, we have shown the significance of the expression of integrins on bladder transitional cell cancer, especially the correlation between VLA-5 and malignant behaviour of TCC cells. Although the functional aspect of these results are still unclear, integrins, especially VLA-5, might have important roles in the invasion and metastasis of transitional cell cancer.

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