Original Articles

Expression of CD44v6 Correlates with Cell Proliferation and Cellular Atypia in Urothelial Carcinoma Cell Lines 5637 and HT1197

(CD44 adhesion molecules / urothelial carcinoma / cell lines / differentiation)

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Abstract. CD44 comprises a family of membrane adhesion molecules encoded by a single gene and diversified by alternative splicing and extensive posttranslational modifications. Alterations of CD44 expression patterns are linked to tumour invasion and formation of metastases. However, CD44 expression and its relation to the biological properties of tumours vary depending on the tumour type and origin. In transitional cell carcinoma of the urinary bladder, low CD44 expression is linked to enhanced tumour aggressiveness. We studied CD44 expression in two urothelial cancer cell lines, HT1197 and 5637. CD44s and a v6 variable exon-containing splice variants were detected in both cell lines by reverse transcription-PCR and by commercially available monoclonal antibodies. In both cell lines, Western blot analysis detected immunoreactive proteins with approximate sizes 70–85 kD, 95–110 kD, and 120–140 kD with CD44v6 antibody and weak bands with size 70–98 kD with CD44s antibody. At the cellular level, the pattern of CD44 immunoreactivity correlated with a lower level of cell differentiation and a higher degree of cell proliferation. In HT1197 cells, the CD44v6 was detected predominantly in small proliferating cells and in large multinuclear atypical cells. CD44s and CD44v6 displayed low immunoreactivity in HT1197 cells with a higher degree of epithelial differentiation. The 5637 cells expressed CD44v6 strongly and CD44s weakly. We conclude that CD44v6 expression correlates with a higher proliferative activity and with a stem cell-like phenotype in both cell lines and with cellular atypia in HT1197 cells.

CD44 forms a family of cell surface adhesion glycoproteins involved in the regulation of organ differentiation and the maintenance of tissue integrity (Borland et al., 1998; Sneath and Mangham, 1998; Goodison et al., 1999). These proteins are also engaged in tissue repair and immune reactions (Seth et al., 1991; Kaya et al., 1997). The CD44 protein molecules ranging in size from 38 kD to approximately 250 kD originate from a single gene residing on human chromosome 11 (11p13) (Screaton et al., 1992). Alternative splicing and subsequent extensive posttranslational modifications including glycosylation, phosphorylation and enzymatic cleavage result in a spectrum of proteins that differ in size as well as in composition of functional domains (Screaton et al., 1992; Kajita et al., 2001).

Alterations in the CD44 expression pattern have been shown to be linked to neoplastic transformation of various cell types and may be closely related to invasive growth and formation of metastases of several tumour types (Gotley et al., 1996; Goodison and Tarin, 1998; Lipponen et al., 1998; Saegusa et al., 1999; Mikami et al., 2000; Nguyen et al., 2000; Khoursheed et al., 2002). The profile of CD44 isoforms is variable in tumours of identical histologic type, depending especially on the degree of differentiation and cytologic atypia of the tumour cells. The CD44 profile is also closely related to the malignant features of tumours (Hong et al., 1995; Sugino et al., 1996; Lipponen et al., 1998; Nguyen et al., 2000; Wong et al., 2003).

The CD44 gene spans almost 92 kb and contains at least 20 exons. CD44 variants contain five constant

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exons on both ends of the mRNA and variable exons (exon 6 to 15) that are positioned in the centre. The translated variable exons form a membrane proximal extracelluar part of the protein that is involved in ligand binding and interaction with other membrane proteins. There are also at least two splice variants of the intracellular part of the protein. Three fundamental functions of CD44 molecules have been identified: 1/ binding to components of the extracellular matrix and extracellular ligands, 2/ modulator function for membrane receptors, and 3/ organization of the actin-containing cytoskeleton (for review see Rudzki and Jothy, 1997, and Ponta et al., 2003). The smallest isoform (CD44s), lacking the variable exon, is expressed in many tissues and cell types, while variant exon-containing forms (CD44v1–10) are restricted to a limited number of tissues and developing organs (Mackay et al., 1994; Sherman et al., 1998). CD44 expression, especially of isoforms containing the variable exons, was found in numerous tumours (Gotley et al., 1996; Goodison and Tarin, 1998; Goodison et al., 1999; Nguyen et al., 2000; Khoursheed et al., 2003). Transitional cell carcinoma of the urinary bladder is one of the most commonly diagnosed human malignancies and its incidence and prevalence are rising. Alterations of expression of several genes, including CD44, associated with the neoplastic transformation of urothelial cells and progression of urothelial carcinomas have been described (Southgate et al., 1995; Sugino et al., 1996; Lipponen et al., 1998; Ioachim et al., 2002). In these carcinomas, a poor prognosis and higher tumour grade are associated with low expression of CD44 molecules (Southgate et al., 1995; Müller et al., 1997, and reviewed in Martin et al., 2003), similar to the endometrial and squamous cancers. The differences in CD44 expression in transitional cell tumours and an easily detected expression in the basal layers of normal urothelium (Southgate et al., 1995; Sugino et al., 1996; Müller et al., 1997) prompted us to search for CD44 expression in urothelial cancer cell lines. Here we report the findings of CD44s and CD44v6 expression in 5637 and HT1197 cell lines that differ in morphology, growth properties and the degree of differentiation of cancer cells.

Material and Methods

Cell culture

HT1197 and 5637 cell lines were obtained from the collection of cell cultures of the Laboratory of Pharmacotoxicology of the National Institute for Cancer Research, Genova, Italy. Both cell lines were maintained under standard culture conditions. HT1197 cells were grown in D-MEM medium (Sevapharma, Prague, Czech Republic) supplemented with 10% foetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% non-essential amino acids (Sigma-Aldrich Chemie, Taufkirchen, Germany). 5637 cells were grown in RPMI medium (Sevapharma) supplemented with 10% foetal bovine serum. Antibiotics, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich Chemie) were added to the culture medium. Both cell lines were maintained in plastic flasks (Nunc, Roskilde, Denmark) at 37°C in humidified atmosphere containing 5% CO_2.

Doubling time determination

Cells were plated at 2.8×10^5 in a 25-cm^2 plastic culture flask (Nunc) and were incubated for 96 h in culture medium as described above. Doubling time calculations were performed by dividing 96 h by the log base 2 of the cell count at time 96 h minus the log base 2 of the cell count at time 0.

Light microscopic evaluation of cell morphology

Cells grown on microscopic slides in special chambers as described elsewhere (Mandys and Elleder, 1980) were fixed with ice-cold methanol and stained with Giemsa. Slides were then observed under an optical microscope (Nikon ECLIPSE E 400).

Immunostaining

Immunohistochemical staining was performed using the peroxidase method by the avidin-biotin complex technique with 3,3' diaminobenzidine (DAB) as the final chromogen. CD44s was detected using mouse monoclonal antibody, clone DF1485 (DAKO, Glostrup, Denmark) in dilution 1 : 50. To detect CD44v6, mouse monoclonal antibody, clone V6-B3 (Zymed Laboratories, San Francisco, CA) in dilution 1 : 100 was used. Cells grown on microscopic slides (see above) were fixed with ice-cold methanol and after drying treated with 0.05% solution of Triton X-100 (Serva, Heidelberg, Germany), washed and incubated in a citrate buffer, pH 7.4, for 40 min at 98° C. Incubation with primary antibodies was performed in a moist chamber for 60 min at room temperature. Antigens were visualised using a DAKO LSAB Kit (DAKO Universal LSAB Kit, DAKO, Carpinteria, CA) and the cells were counterstained using Mayer's haematoxylin. The stained slides were evaluated semi-quantitatively according to the quantity and distribution of positive cells in both cell lines, using an optical microscope (Nikon ECLIPSE E 400).

Reverse transcription, PCR and sequencing

Primers were designed according to the CD44 cDNA (NM_000610) and gene (NT_009237.16) and were as follows: P1s: (33965871)5'GAC ACA TAT TGC TTC AAT GCT TC; P2s: (33972328) 5'GAA GAT TGT ACA TCA GTC ACA GAC; P3s: (33989999)5'TCC AGG CAA CTC CTA GTA GTA CAA; P4a: (33990127)5'TGT CCC TGT TGT CGA ATG GGA GTC; P5a: (34007219)5'CTG TTG ACT GCA ATG CAA ACT GCA ;

P6a: (34014818) 5' TTA CAC CCC AAT CTT CAT GTC CA (numbers indicate the position of 5' nucleotide in the sequence (NT_009237.16).

Cells were washed three times with PBS, scraped using a cell lifter and centrifuged for 10 min at 170 × *g* in a Hettich 32R centrifuge. Total RNA was prepared using TRIZOL (Invitrogen, Carlsbad, CA) and 2 µg of total RNA were used for reverse transcription with SuperScriptII reverse transcriptase (Invitrogen) and random hexamers. Two µl of reverse transcription mixture were used for subsequent amplification by polymerase chain reaction using Taq polymerase (Invitrogen) and PTC-200 termocyclers (MJ Research, Waltham, MA). The termocycling protocol consisted of melting at 95°C for 1 min, followed by 10 cycles: 94°C 1 min, 62°C 1 min, 72°C 3 min; 15 cycles: 94°C 1 min, 60°C 1 min, 72°C 3 min, and 10 cycles: 94°C 1 min, 68°C 1 min, 72°C 3 min. For nested PCR, 5 µl of PCR mixture and one inner primer were used for a subsequent round of amplification. Amplified fragments were resolved by agarose gel electrophoresis, stained by ethidium bromide, visualized by a UV transluminator and recorded by a digital camera.

To confirm the specificity of CD44v6 amplification, the amplified fragments were eluted from electrophoretic gels and sequenced directly using gene-specific primers in an ABI Prism 3100-Avant capillary sequencer (Applied Biosystems, Foster City, CA).

Western blot analysis

For **Western blot** analysis, both cell lines were grown in 75 cm^2 tissue culture flasks, washed three times in PBS, removed using a plastic cell lifter and centrifuged for 10 min at 170 × *g* in a Hettich 32R centrifuge. Protein extracts were prepared using a standard protocol with Laemmli buffer. Protein content was estimated using a BCA Protein Assay Kit (Pierce, Rockford, IL). Twenty µg of protein were loaded per lane, separated by electrophoresis in TRIS/SDS 10% polyacrylamide gel in a Mini Protean II apparatus (Bio-Rad Laboratories, Hercules, CA). Separated proteins were electro-transferred on PVDF membrane.

Membranes were pre-incubated in buffer PBT (PBS containing 0.1% Tween 20 (Sigma, St. Louis, MO)) containing 5% (w/v) nonfat dry milk (overnight, 4°C). For immunodetection, CD44s antibody (clone DF1485, DAKO, Glostrup, Denmark) and CD44v6 antibody (Zymed Laboratories, San Fracisco, CA) were used at a dilution ranging from 1 : 1000 to 1 : 4000 in PBT with 5% (w/v) milk. Incubations in primary antibodies were carried out at room temperature for 60 min on a rocking plate. Membranes were washed 6 times in PBT for a total time of 60 min and incubated with goat anti-mouse IgG antibodies labelled by peroxidase (Sigma) diluted in PBT (1 : 10 000 or 1 : 20 000) for 60 min at room temperature and subsequently washed six times in PBT for a total time of 60 min. The ECL plus chemiluminescent system (Amersham/Pharmacia, Uppsala, Sweden) was used for detection as recommended by the supplier. For densitometric analysis, films were scanned, saved in TIFF format and analyzed in triplicates using the program NIH image in the PC version (http://rsb.info.nih.gov/nih-image/).

Results

I. Characterization of HT1197 and 5637 cell lines

Cell morphology. Morphological examination at the light microscopic level showed that the HT1197 cell line was composed of a heterogenous population represented by three main cell types: A – small spindle-shaped or polygonal cells with minimal cytoplasm and a high nuclear-cytoplasmic ratio representing a less differentiated phenotype. These cells frequently formed a network within the other cell types. Type B cells were morphologically characterized by an epithelial appearance with a relatively large cytoplasm, predominantly polygonal or triangular shape, and a low nuclear-cytoplasmic ratio. These type B cells often formed solid nests surrounded by the cells of type A. Type C represented giant cells, containing a large polyploid nucleus or multinucleated (Fig. 1a).

Cell line 5637 was composed only of relatively uniform undifferentiated small round-shaped or short spindle-shaped cells with scanty cytoplasm and a high nuclear-cytoplasmic ratio (Fig. 1b).

Growth properties. The doubling time of HT1197 cells was 40 h, while the doubling time of 21.9 h was obtained for 5637 cells. This difference indicates a higher proliferative activity of 5637 cells, consistent with their smaller size and immature phenotype. Microscopic examination of cells plated at low densities was in keeping with the faster proliferation of 5637 cells and small cells in HT1197 cultures. In HT1197, the apparently fast dividing cells correlated with the type A.

II. Expression of CD44s and CD44v6 isoforms in HT1197 and 5637 cell lines

Detection of CD44s and CD44v6 mRNA. For detection of CD44 transcripts, primers corresponding to 5' and 3' constant regions of the CD44 mRNAs were designed as well as for the exon that is part of the CD44v6 variant (Fig. 2). RT-PCR resulted in multiple amplified fragments. The pattern of amplified fragments supported the expression of CD44v6 (Fig. 3 panels a and b, lanes 1, 2 and 6; Fig. 3c lanes 1, 3, 5 and 7) and CD44s in both cell lines (Fig. 3a and b lanes 3 and 4, Fig. 3c lanes 2, 4, 6 and 8). PCR with primers specific for the v6 variable exon and constant exons on both sides of the cDNA amplified multiple fragments, presumably corresponding to splice variants containing the v6 exon and other variable exons positioned on both sides of the v6 exon (Fig. 3a lane 1 (marked by asterisks), Fig. 3a and b lanes 5 (marked as v6–10)). Primers

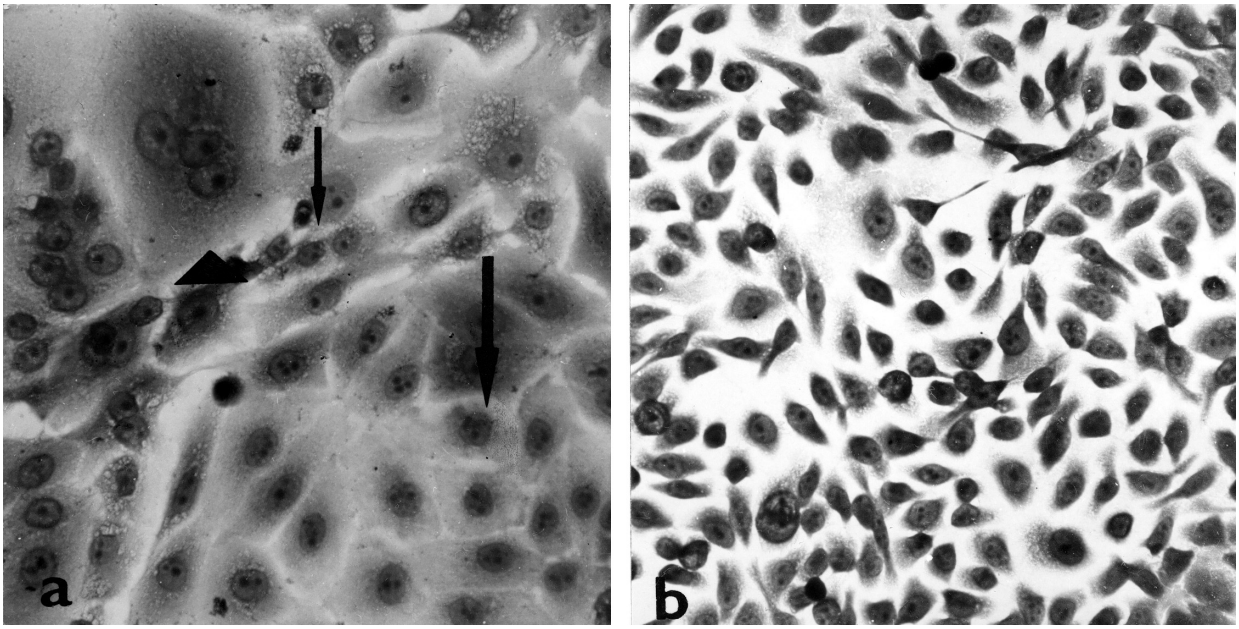

Fig. 1. Morphological analysis of cultured cells. a – HT1197 cells display prominent morphological diversity: small spindle-shaped cells of type A (small arrow), large cells of epithelial appearance – type B (big arrow) and giant multinuclear cells – type C (arrowhead). b – Cell line 5637 is composed of small uniform predominantly spindle-shaped cells. Giemsa staining, 200x.

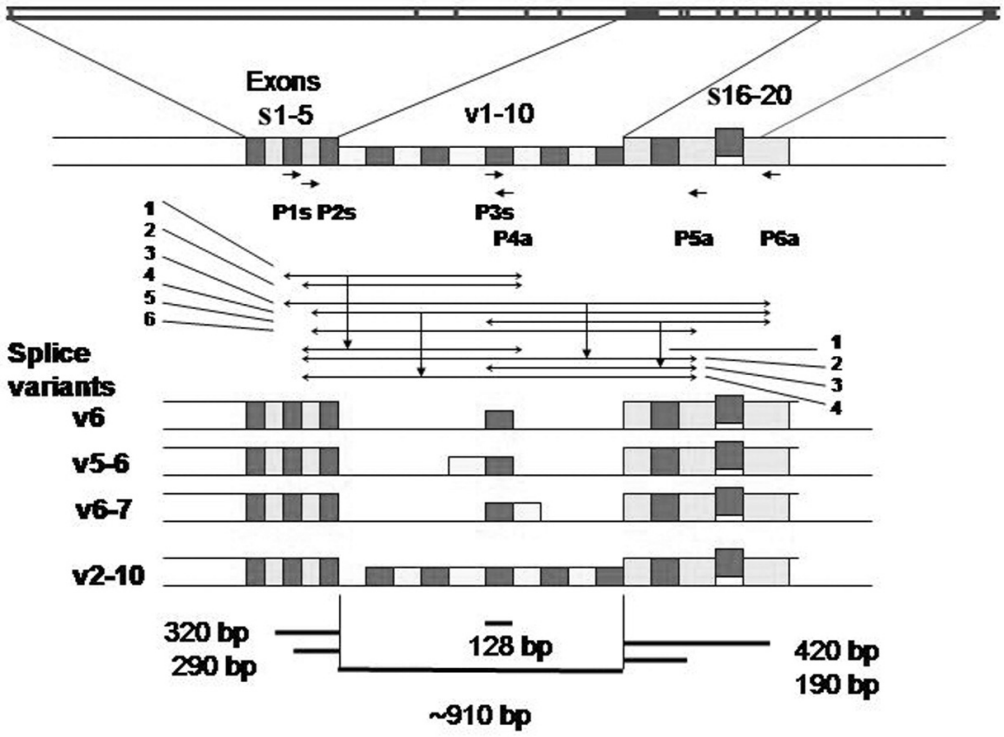

Fig. 2. Schematic representation of the CD44 gene. The gene spans 92 kb and contains at least 20 regular exons. Standard exons are marked as s1–5 (in the 5'part of the gene) and s16–20 (in the 3' part of the gene). The gene organization is derived from GenBank accession number NT_009237.16. Variable exons are numbered as v1–10 and correspond to exons 6–15. Arrows represent primers P1s, P2s, P3s in the sense direction and P6a, P5a, and P4a in the antisense direction. Double head arrows represent regions that allow identification of CD44s and CD44v6 exon-containing variants. Splice variants v6, v5–6, v6–7, and v2–10 are indicated in the lower part of the figure.
Scheme for primary PCR (numbered 1–6) is indicated in the left part of the figure and the secondary (nested) PCR (numbered 1–4) is indicated in the right part of the figure. Vertical arrows indicate the strategy for the nested PCR. Sizes of expected fragments that may be amplified with primers used in this study are given in the lower part of the figure.

designed to amplify the long version of the intracellular part of CD44 amplified preferentially CD44s in both cell lines (Fig. 3a and b lanes 3). Primers designed to amplify CD44 from extracellular constant exons to the transmebrane domain amplified preferentially the CD44v6, indicating the prevalence of CD44v6 in both cell lines (Fig. 3a and b lanes 6). Additional CD44 transcripts containing the v6 exon were amplified using primers specific for the v6 exon and the long variant of the CD44 intracellular domain. In both cell lines, the most prevalent variant of this type corresponded to variants containing v6–10 variable exons (Fig. 3a and b lanes 5). Contrary to that, primers specific for the extracellular constant exons and the transmembrane domain amplified CD44v6 containing a single variable exon (Fig. 3 panels a and b, lanes 6). The specificity of the CD44v6 isoform was confirmed by direct sequencing of amplified fragments.

Western blot analysis. Western blot analysis using antibodies specific for CD44s and CD44v6 detected immunoreactive proteins in both cell lines. The antibody specific for CD44s detected immunoreactive proteins with approximate sizes of 70–98 kD. The antibody specific for CD44v6 recognized proteins of 70–85 kD, 95–110 kD, and 120–140 kD. The expression of the v6 exon-containing variants were the most abundant in both cell lines (Fig. 4). HT1197 expressed a markedly larger amount of CD44v6 immunoreactive proteins in the position approximately 110 kD compared to 95–98 kD. The ratio of modified 110 kD CD44v6/95–98 kD CD44v6 was 1.18 +/- 0.12 in HT1197 cells versus 0.76 +/- 0.06 in 5637 cells. This presumably corresponds to a higher level of post-translational modification of the CD44v6 protein in HT1197 cells. Immunoreactive proteins migrating at 70–98 kD and corresponding to CD44s were less abundant in both cell lines.

CD44s antibody does not recognize the prominent bands detected by the CD44v6 antibody (migrating at position 98–110 kD), consistent with the specificity of the CD44v6 antibody. Nevertheless, prominent bands with approximate size 40 kD and 58 kD were detected by both antibodies and were also slightly visible in controls, where the primary antibody was omitted. The band with approximate size 40 kD was more prominent in HT1197 cells. Proteins with an approximate size 70–98 kD were detected solely by CD44s antibody and were visible in films exposed for longer periods of time.

III. Correlation of CD44 expression with cell differentiation in HT1197 and 5637 cell lines

CD44 immunostaining. Both CD44s and CD44v6 splicing variants were detected immunohistochemically in HT1197 and 5637 cells. In the HT1197 cell line, the intensity of immunostaining was heterogenous and differed depending on the cell type (A, B or C). CD44v6 was positive in the majority of type A cells. Positive staining was often pronounced at the edges of the cells

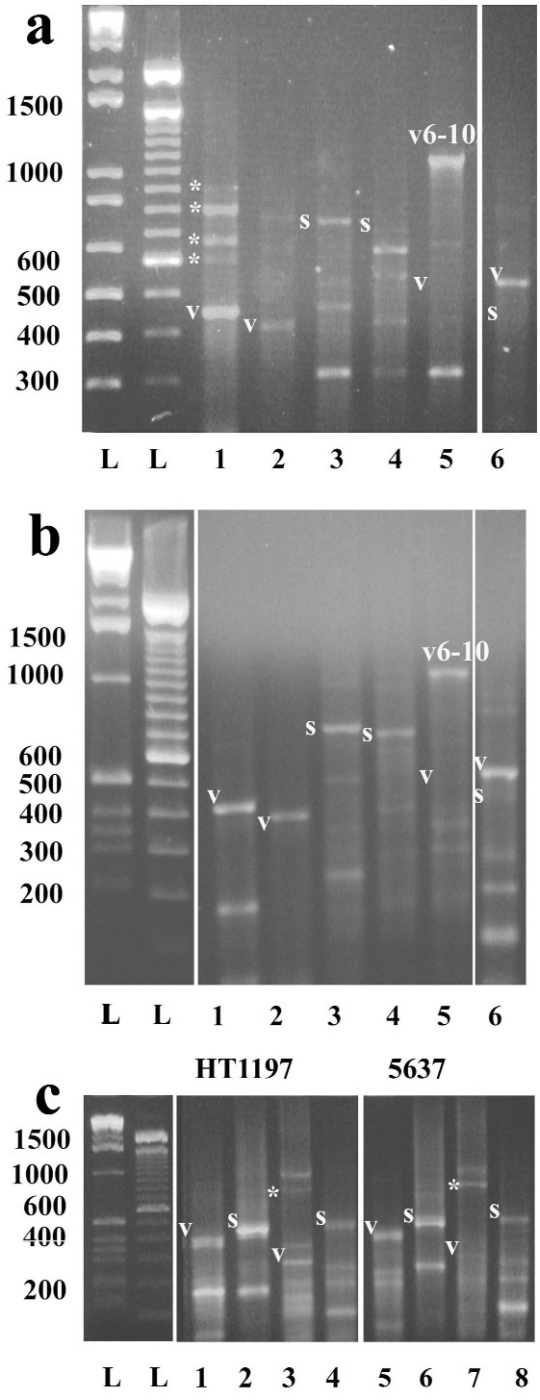

Fig. 3. Detection of CD44 transcripts by reverse transcription and polymerase chain reaction. (a) – Amplification of CD44v6 and CD44s fragments from HT1197 cells and (b) – from 5637 cells. Fragments corresponding to amplified regions of CD44v6 and CD44s are indicated as v and s, respectively. (c) – Nested PCR amplification indicating the expression of CD44v6 (v) and CD44s (s). Lanes 1–4 represent fragments of CD44v6 and CD44s amplified from HT1197 cells and lanes 5–8 represent fragments of CD44v6 and CD44s amplified from 5637 cells and correspond to the reaction scheme indicated in Fig. 2 as 1 to 4, respectively, for each cell line.

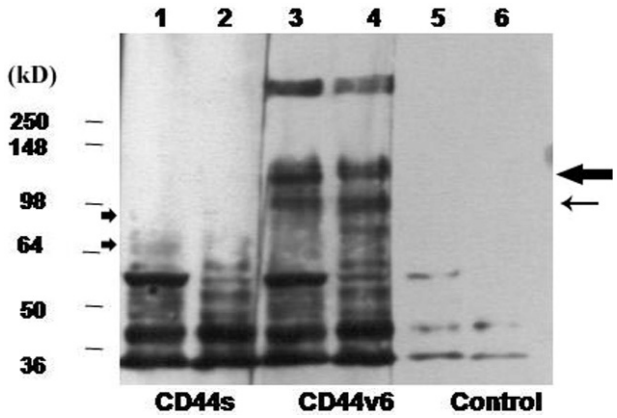

Fig. 4. Western blot analysis of protein extracts from HT1197 (lanes 1, 3, 5) and 5637 cells (lanes 2, 4 and 6) with antibodies directed against CD44s (lanes 1 and 2) and CD44v6 (lanes 3 and 4). Lanes 5 and 6 are controls in which the primary antibodies were omitted. Arrows indicate the position of presumably post-traslationally modified (big arrow) and unmodified (small arrow) CD44v6. CD44s is detected as faint bands indicated by short arrows. Proteins detected by both antibodies in position 38 kD–58 kD are also weakly detected in controls by secondary antibody and may be non-specific or represent proteins containing both CD44s and CD44v6 epitopes.

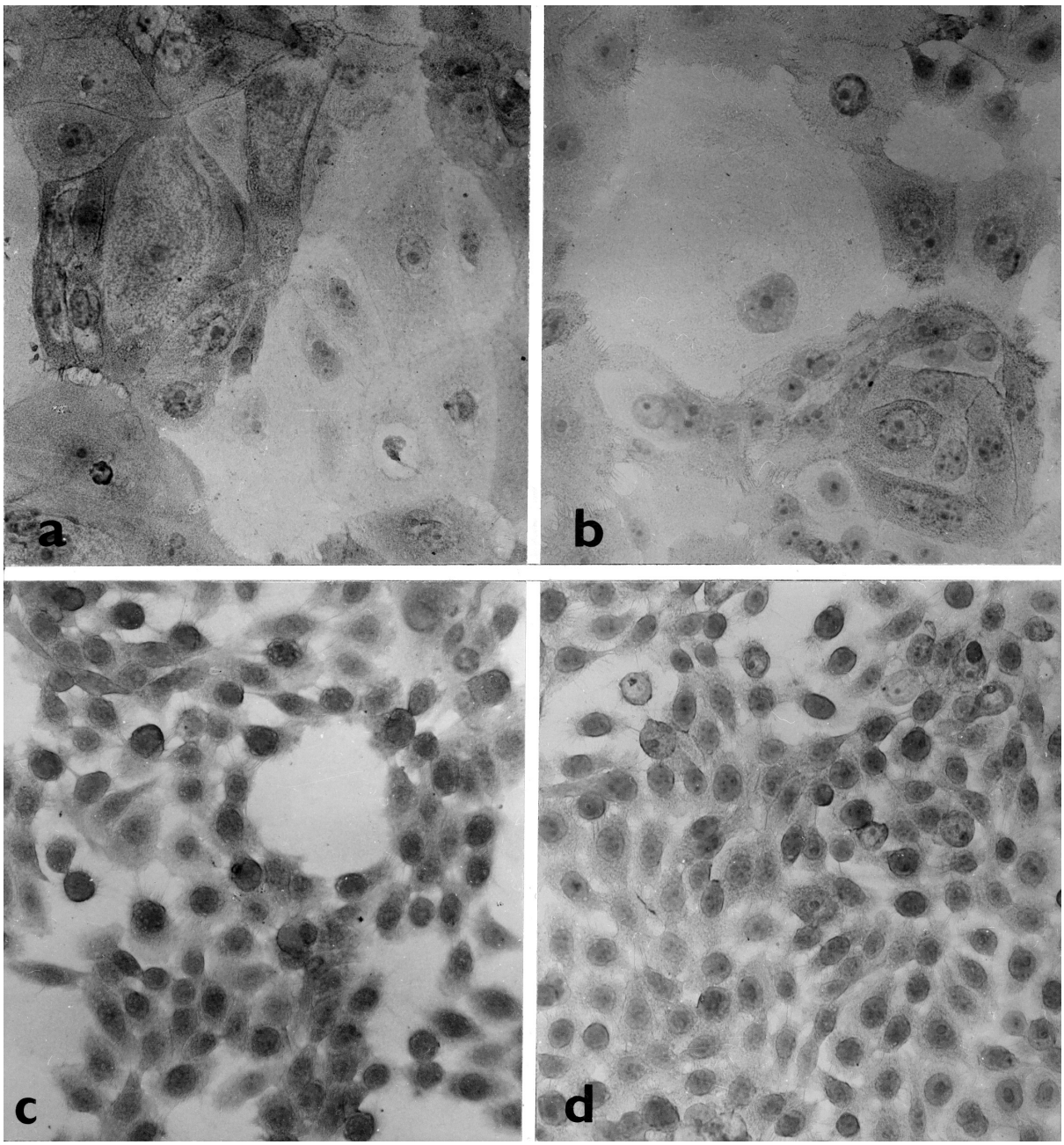

Fig. 5. Analysis of CD44v6 and CD44s expression at the cellular level. Cell line HT1197 (panels a and b): a – immunostaining for CD44v6 in cells of type A and C. The group of type B cells is CD44v6-negative. b – Immunostaining for CD44s is positive in type B cells, negative in the cells of type C and weakly positive in some cells of type A. Cell line 5637 (panels c and d): c – regular positive immunostaining of CD44v6 and d – weak immunoreactivity for CD44s. 200×.

in keeping with the membrane localization of both epitopes. Relatively strong imunoreactivity for CD44v6 was also present in type C cells. Weak or even negative staining was observed in the majority of type B cells. Immunoreactivity for the CD44s molecule was detected in a portion of type B cells and in some cells of type C. A high portion of A type cells and some C type cells were CD44s negative (Fig. 5a, b and Table 1).

In 5637 cells, immunostaining for both antigens resulted in a uniform and diffuse pattern. Strong staining was observed for the CD44v6 and relatively weak staining for CD44s (Fig. 6c, d and Table 2).

Table 1. Semi-quantitative evaluation of CD44v6 and CD44s detection in HT1197 cells. Comparison of CD44 expression in individual cell types A, B and C

Cell type	A	B	C
Cell characteristics	Small polygonal cells, low grade of differentiation	Large cells with epithelial characteristics	Giant multinuclear cells
CD44v6 detection	Strong (+++)	Weak or missing (+/–)	Relatively strong (++)
CD44s detection	Weak or missing (+/–)	Relatively strong, partly negative (++/+/–)	Weak or missing (+/–)

Table 2. Semi-quantitative evaluation of CD44v6 and CD44s detection in 5637 cells

Detected antigen	
CD44v6	Diffuse moderate staining (++)
CD44s	Diffuse weak staining (+)

Discussion

Members of the CD44 protein family play important roles in cell adhesion, cell motility, cytoskeleton organization, cell recognition and modulation of signal transduction pathways. The aim of the present study was to characterize CD44s and CD44v6 expression in two different cell lines established from urinary bladder carcinomas and to compare the results with morphological and growth properties of these two cell lines.

Our results indicate that multiple CD44 variants are expressed in the urinary carcinoma cell lines HT1197 and 5637. The expression of CD44s and CD44v6 is supported by the results of RT-PCR in both cell lines and the specificity was confirmed by sequencing of amplified fragments. In both cell lines, CD44s was detected with a preference for isoforms containing the long version of the intracellular domain. However, RT-PCR reactions directed to amplify CD44 from the constant extracellular domain to the transmembrane domain preferentially detected CD44v6 in both cell lines, suggesting that expression of CD44v6 is higher than that of CD44s. Nevertheless, the preferentially amplified CD44 variants containing the v6 variable exon and long form of the intracellular domain are variants containing several variable exons. Although the pattern of amplified fragments is similar in both cell lines, there were differences. The more differentiated HT1197 cell line expressed four v6-containing variants with additional variable exons in the region v2–v5 (Fig. 3a, lane 1). The nested PCR amplified preferentially CD44v6 in both lines, suggesting that the differences in the extracellular part of CD44 may be based on alternative splicing in presumably constant exons.

Antibodies detected CD44s and CD44v6 immunoreactive proteins in both cell lines in Western blots. While 40 kD- and 58 kD-sized proteins were recognized by both CD44s and CD 44v6 antibodies, the results support the specific detection of CD44s proteins with a size of 70–98 kD. Proteins corresponding to 70–85 kD, 95–110 kD, and 120–140 kD were recognized by the CD44v6 antibody in both cell lines. Densitometric analyses indicated a larger proportion of higher post-translationally modified CD44v6 proteins (migrating in the range of 95–140 kD) in HT1197 cells compared to 5637 cells. Since glycosylation critically affects ligand binding and other functions of CD44 (Skelton et al., 1998), it is possible that the observed differences between 5637 and HT1197 cells reflect the differentiation process in the latter cell line.

Proteins with the size 40 kD and 58 kD were recognized by both antibodies in Western blot analyses. It seems likely that the detection is non-specific since these bands were recognized weakly by the secondary antibody. Nevertheless, it cannot be excluded that the strongly labelled bands in the range of 40 kD and 58 kD may correspond to CD44 variants bearing both CD44s and CD44v6 epitopes.

The detection of CD44s and CD44v6 by immunocytochemistry was consistent with results obtained by Western blots. Our findings document that expression of both CD44s and the splice variant CD44v6 was dependent on the phenotype that correlated with the differentiation status of cultured cells. CD44 proteins showed regular positive immunoreactivity in uniform undifferentiated 5637 cells. In contrast, HT1197 cells having a more differentiated epithelial phenotype, but much more prominent cytological irregularities, displayed substantial differences in the expression of CD44 molecules. Previous studies of the histological slides of both normal and neoplastic urothelium have shown that immunoreactivity for CD44 was located mainly in less differentiated cells forming the basal layer of normal

transitional epithelium (Southgate et al., 1995; Sugino et al., 1996). These cells display the properties of tissue progenitor (stem) or transit-amplifying cells: an undifferentiated phenotype, a higher proliferative activity and the ability to change into the more differentiated phenotype of the superficial layers of the urothelium. Differentiated superficial cells of normal urothelium do not display CD44 immunoreactivity. In differentiated papillary urothelial carcinomas, CD44-positive cells extend into the superficial compartment of neoplastic urothelium (Sugino et al., 1996). This phenomenon probably reflects abnormal differentiation of basal stem and/or transit-amplifying cells and their shift into the superficial layers of the neoplastic urothelium, with a corresponding increase in immunoreactivity for CD44 in these carcinomas. In our study, cell line 5637 revealed morphological and growth properties of stem cells, that is an undifferentiated phenotype, uniform cytological appearance and high proliferative activity. Regular expression of CD44 molecules observed in our study and expression of p63 published by Urist et al. (2002) support the stem cell character of the 5637 line.

In high-grade urothelial carcinomas, either flat, or those invading the wall of the urinary bladder, prominent irregularities in CD44 expression, or even its absence, have been described (Hong et al., 1995; Sugino et al., 1996; Toma et al., 1999). In our study, substantial diversity of CD44 expression was observed in the HT1197 cell line, derived from invasive transitional cell carcinoma. Distinct CD44-positive and CD44-negative cell populations were also described in the HT1197 cell line in one previous study (Southgate et al., 1995). The cytologic appearance of the HT1197 cell line corresponds with a high-grade carcinoma. In these tumours, a high degree of cytological atypia is related to abnormal cell differentiation, reflecting a high degree of neoplastic transformation. Such alterations in urothelial carcinomas are frequently connected with altered CD44 expression and highly aggressive tumour, clinically manifested by higher malignancy. Similar differences in the expression of CD44 molecules we described in our previous study in lung carcinoid tumours. While in typical carcinoid tumours showing uniform cytological appearance the expression of CD44s and CD44v6 was strong and regular, negative immunostaining was found in cytologically irregular atypical carcinoids (Nguyen et al., 2000).

The results of our present study may contribute to the elucidation of the heterogeneity of CD44 expression in urothelial carcinomas. The alterations of CD44 molecules are closely related to the carcinoma grade. The decrease or absence of CD44 expression is not linked to the undifferentiated cell phenotype, corresponding with tissue progenitor (stem) or transit-amplifying cells, but it is likely to be linked to the derangement of differentiation of tumour cells, morphologically manifested by prominent cytological atypia in high-grade urothelial carcinomas.

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References

Borland, G., Ross, J. A., Guy, K. (1998) Forms and functions of CD44. *Immunology* **93,** 139-148.

Goodison, S., Tarin, D. (1998) Current status of CD44 variant isoforms as cancer diagnostic markers. *Histopathology* **32,** 1-6.

Goodison, S., Urquidi, V., Tarin, D. (1999) CD44 cell adhesion molecules. *J. Clin. Pathol. Mol. Pathol.* **52,** 189-196.

Gotley, D. C., Fawcet, J., Walch, M. D., Reeder, J. A., Simmons, D. L., Antalis, T. M. (1996) Alternatively spliced variants of the cell adhesion molecule CD44 and tumour progression in colorectal cancer. *Br. J. Cancer* **74,** 342-351.

Hong, R. L., Pu, Y. S., Hsieh, T. S., Chu, J. S., Lee, W. J. (1995) Expressions of E-cadherin and exon v6-containing isoforms of CD44 and their prognostic values in human transitional cell carcinoma. *J. Urol.* **153,** 2025-2028.

Ioachim, E., Charchanti, A., Stavropoulos, N., Athanassiou, E., Bafa, M., Agnantis, N. J. (2002) Expression of cathepsin D in urothelial carcinoma of urinary bladder: an immunohistochemical study including correlations with extracellular matrix components, CD44, p53, Rb, c-erbB2 and the proliferation indices. *Anticancer Res.* **22,** 3383-3388.

Kajita, M., Itoh, Y., Chiba, T., Mori, H., Okada, A., Kinoh, H., Seiki, M. (2001) Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. *J. Cell. Biol.* **153,** 893-904.

Kaya, G., Rodriguez, I., Jorcano, J. L., Vassalli, P., Stamenkovic, I. (1997) Selective suppression of CD44 in keratinocytes of mice bearing an antisense CD44 transgene driven by a tissue-specific promoter disrupts hyaluronate metabolism in the skin and impairs keratinocyte proliferation. *Genes Dev.* **11,** 996-1007.

Khoursheed, M., Mathew, T. C., Makar, R. R., Sonia, L., Abul, H., Asfar, S., Al-Sayer, H., Dashti, H. M., Al-Bader, A. (2002) Expression of CD44s in human colorectal cancer. *Path. Oncol. Res.* **8,** 170-174.

Lipponen, P., Aaltoma, S., Kosma, V.-M., Ala-Opas, M., Eskelinen, M. (1998) Expression of CD44 standard and variant-v6 proteins in transitional cell bladder tumours and their relation to prognosis during a long-term follow-up. *J. Pathol.* **186,** 157-164.

Mackay, C. R., Terpe, H. J., Stauder, R., Marston, W. L., Stark, H., Gunthert, U. (1994) Expression and modulation of CD44 variant isoforms in humans. *J. Cell. Biol.* **124,** 71-82.

Mandys, V., Elleder, M. (1980) Demonstration of enzymes in cells cultured on semipermeable membrane in a double chamber. *Histochemistry* **65,** 325-327.

Martin, T. A., Harrison, G., Mansel, R. E., Jiang, W. G. (2003) The role of the CD44/ezrin complex in cancer metastasis. *Crit. Rev. Oncol. Hematol.* **46,** 165-186.

Mikami, T., Mitomi, H., Hara, A., Yanagisawa, N., Yoshida, T., Tsuruta, O., Okayasu, I. (2000) Decreased expression of CD44, alpha-catenin, and deleted colon carcinoma and altered expression of beta-catenin in ulcerative colitis-associated dysplasia and carcinoma, as compared with sporadic colon neoplasms. *Cancer* **89,** 733-740.

Müller, M., Heicappell, R., Habermann, F., Kaufmann, M., Steiner, U., Miller, K. (1997) Expression of CD44V2 in transitional cell carcinoma of the urinary bladder and in urine. *Urol. Res.* **25,** 187-192.

Nguyen, V. N., Mirejovsky, T., Melinova, L., Mandys, V. (2000) CD44 and its v6 spliced variant in lung carcinomas: Relation to NCAM, CEA, EMA and UP1 and prognostic significance. *Neoplasma* **47,** 400-408.

Ponta, H., Sherman, L., Herrlich, P. A. (2003) CD44: from adhesion molecules to signalling regulators. *Nat. Rev. Mol. Cell. Biol.* **4,** 33-45.

Rudzki, Z., Jothy, S. (1997) CD44 and the adhesion of neoplastic cells. *J. Clin. Pathol.* **50,** 57-71.

Saegusa, M., Hashimura, M., Machida, D., Okayasu, I. (1999) Downregulation of CD44 standard and variant isoforms during the development and progression of uterine cervical tumours. *J. Pathol.* **187,**173-83.S

Screaton, G. R., Bell, M. V., Jackson, D. G., Cornelis, F. B., Gerth, U., Bell, J. I. (1992) Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. *Proc. Natl. Acad. Sci. USA* **89,** 12160-12164.

Seth, A., Gote, L., Nagarkatti, M., Nagarkatti, P. S. (1991) T-cell-receptor-independent activation of cytolytic activity of cytotoxic T lymphocytes mediated through CD44 and gp90MEL-14. *Proc. Natl. Acad. Sci. USA* **88,** 7877-7881.

Sherman, L., Wainwright, D., Ponta, H., Herrlich, P. (1998) A splice variant of CD44 expressed in the apical ectodermal ridge presents fibroblast growth factors to limb mesenchyme and is required for limb outgrowth. *Genes Dev.* **12,** 1058-1071.

Skelton, T. P., Zeng, C., Nocks, A., Stamenkovic, I. (1998). Glycosylation provides both stimulatory and inhibitory effects on cell surface and soluble CD44 binding to hyaluronan. *J. Cell. Biol.* **140,** 431-446.

Sneath, R. J. S., Mangham, D. C. (1998) The normal structure and function of CD44 and its role in neoplasia. *J. Clin. Pathol. Mol. Pathol.* **51,** 191-200.

Southgate, J., Trejdosiewicz, L., Smith, B., Selby, P. (1995) Patterns of splice variant CD44 expression by normal human urothelium *in situ* and *in vitro* and by bladder-carcinoma cell lines. *Int. J. Cancer* **62,** 449-456.

Sugino, T., Gorham, H., Yoshida, K., Bolodeoku, J., Nargund, V., Cranston, D., Goodison, S., Tarin, D. (1996) Progressive loss of CD44 gene expression in invasive bladder cancer. *Am. J. Pathol.* **149,** 873-882.

Toma V., Hauri, D., Schmid U., Ackermann, D., Maurer, R., Alund, G., Knönagel, H., Rist, M., Gasser, T.C., Sauter, G., Roth, J. (1999) Focal loss of CD44 variant protein expression is related to recurrence in superficial bladder carcinoma. *Am. J. Pathol.* **155,** 1427-1432.

Urist, M. J., Di Como, C. J., Lu, M-L., Charytonowicz, E., Verbel, D., Ince, T. A., McKeon, F. D., Cordon-Cardo, C. (2002) Loss of p63 expression is associated with tumor progression in bladder cancer. *Am. J. Pathol.* **161,** 1199-1206.

Wong, K., Rubenthiran, U., Jothy, S. (2003) Motility of colon cancer cells: modulation by CD44 isoform expression. *Exp. Mol. Pathol.* **75,** 124-130.