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Immunoelectron microscopic demonstration of the membrane proteases aminopeptidase N/CD13 and dipeptidyl peptidase IV/CD26 in normal and neoplastic renal parenchymal tissues and cells

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SUMMARY

Aminopeptidase N (APN, CD13) and dipeptidyl peptidase IV (DPP IV, CD26) are transmembrane ectoenzymes occurring in a wide variety of cells. They are involved in tumour cell invasion and the formation of metastases. A basis for further information about these enzymes is the exact ultrastructural localization in normal and malignant cells. In this paper, we demonstrate the precise subcellular localization of the membrane peptidases APN and DPP IV on the cell surfaces in renal tissues, renal cell carcinoma, cultured renal parenchymal cells and cultured renal carcinoma cells. Using cryo-ultramicrotomy of weakly fixed tissues and cells in combination with indirect immunogold labelling, both membrane peptidases were detectable on the external cell surfaces. They showed different ultrastructural expression patterns. Both membrane peptidases were abundantly labelled on the external cell surfaces of human kidney proximal tubular cells. The expression pattern of APN/CD13 and DPP IV/CD26 in single labelling was confirmed by a successive double labelling technique. The immunolabelling of CD13 on cultured renal parenchymal cells showed a stronger expression then in cells *in vivo*, but CD26 could not be found. In renal cell cancer (mixed clear cell/chromophilic, poorly differentiated and clear cell type, moderately differentiated) CD13 and CD26 were labelled as in benign renal tissue, but CD26 appeared overexpressed. On the renal carcinoma cells Caki-1 and Caki-2, only one of the two peptidases could be found. CD13 was present non-homogeneously in Caki-1, where the enzyme appeared to form clusters. When CD26 on the cultured renal carcinoma cells Caki-2, is compared with renal proximal tubular cells and renal carcinoma cells in tissue sections, a reduced expression is observed. CD13 was not detected in Caki-2, and CD26 was not found in Caki-1. These small changes on the cell surfaces can only be detected by electronmicroscopic methods. The differences in the distribution of APN/CD13 and DPP IV/CD26 in normal and malignant cells are discussed in connection with literature. Further investigations, especially labelling studies on other neoplastic tissues and cells, will be necessary in order to explain the precise role these membrane peptidases in malignancies.

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INTRODUCTION

Aminopeptidase N (APN, CD13, EC 3.4.11.2) and dipeptidyl peptidase IV (DPP IV, CD26, EC 3.4.14.5) are ubiquitously occurring membrane-bound exopeptidases. They are integral, asymmetrically orientated glycoproteins of the plasma membrane and expose their catalytic sites only at the external surface of the cells. Sequence comparisons of the cloned cDNA showed that APN is identical with the CD13 antigen (Look *et al.* 1989), while DPP IV corresponds to CD26 (Ulmer *et al.* 1990). Both enzymes show species- and organ-dependent differences (McDonald and Barrett 1986). Especially the brush border membranes of renal tubular cells, but also small intestinal enterocytes contain an abundance of APN- and DPP IV-activity. They participate there in the luminal hydrolysis of oligopeptides (McDonald and Barrett 1986). Both enzymes may have also specific roles in the control of growth and differentiation in both hemopoietic and epithelial cell systems (Kenny *et al.* 1989).

In recent years, the interest in cell surface peptidases has increased considerably because, among other things, several reports indicate the involvement of APN and DPP IV in tumour cell invasion and the formation of metastases (Fujii *et al.* 1995, Johnson *et al.* 1993, Menrad *et al.* 1993, Saiki *et al.* 1993). Proteases play an essential part in the proliferative, invasive, and metastasizing behaviour of malignant tumours resulting particularly from their destructive effect on the extracellular matrix (Liotta 1986). Other findings point to an influence of proteases on cell division rate, tumour induction, and tumour promotion (Kennedy and Little 1978).

In order to gain further information on the role of peptidases, it is necessary to find out their precise subcellular location (Gossrau 1985). Most of the papers published on this subject are based on light microscopical enzyme histochemical and immunohistochemical methods. However, with these techniques it is difficult to obtain information about the ultrastructural expression pattern of peptidases. Electron microscopy seems to be best suited to localize macromolecules at the ultrastructural level (Bendayan *et al.* 1987). Until now electron microscopical techniques for ectopeptidase investigations have hardly been applied, which can be explained by methodical problems (Gossrau 1993). Because of their position on the external cell surface, it is difficult to find out the exact localization of ectoenzymes such as APN and DPP IV with conventional immuno-ultrastructural methods (Kettmann *et al.* 1992). Pre- and postembedding methods damage the antigenicity of APN and DPP IV and therefore sufficient labelling is no longer possible (Kettmann *et al.* 1992, Stange *et al.* 1996).

Using cryo-ultramicrotomy of weakly fixed tissues and cells in combination with indirect immunogold labelling, APN and DPP IV are detectable both with single and double labelling in some tissues and cultured cells (Kettmann *et al.* 1992, Stange *et al.* 1996, 1998). This method represents a useful compromise between the preservation of antigenicity and ultrastructural morphology (Slater 1993) and has been proven to be an excellent procedure for the visualisation and localization of cell surface and intracellular proteins (Mizuno 1993). We were interested in the precise ultrastructural demonstration of APN and DPP IV because they have been considered to be involved in cell adhesion processes and metastasis. DPP IV can bind to fibronectin and collagen (Hanski *et al.* 1988). Since the peptidase cleaves peptide bonds at proline, it could be involved in collagen breakdown. Similarly, APN has been described as being closely associated with extracellular matrix components on melanoma cells (Menrad *et al.* 1993). Furthermore, recent studies suggest a role for APN in tumour cell adhesion, invasion and extracellular matrix degradation (Menrad *et al.* 1993, Saiki *et al.* 1993).

In this paper, we wished to demonstrate the precise ultrastructural localization of the membrane peptidases APN and DPP IV to the cell surfaces in renal tissues, renal cell carcinoma, cultured renal parenchymal cells and cultured renal carcinoma cells. Immunoelectron microscopic single and double labelling was in particular performed to study characteristics in the subcellular distribution of both peptidases on the cell surfaces of renal carcinoma cells compared with normal cells.

MATERIALS AND METHODS

Preparation of tissues and cultured cells

Small pieces of fresh human kidney cortex and renal cell cancer (mixed clear cell/chromophilic,

poorly differentiated) (both operation specimens) were washed with ice-cold PBS (pH 7.4) and fixed in 2% formaldehyde and 0.02% glutaraldehyde in PBS for 30 min at room temperature. The investigated cells were the human renal tubular epithelial cell line NP 33 (prepared from nephrectomy tissues as described by Riemann *et al.* (1995)) and the human renal carcinoma cell lines Caki-1 (ATCC) and Caki-2 (ATCC) (gift of Dr. D. Riemann, Institute of Medical Immunology, University of Halle-Wittenberg). After centrifugation and washing in PBS, the cells were just as fixed the tissues, carefully washed in PBS and subsequently embedded in 5-7% gelatine (Sigma, St. Louis, USA) in PBS. Small cell-gelatine pieces were postfixed in 2% formaldehyde and 0.02% glutaraldehyde in PBS for 5 min at 4°C.

All fixed samples (tissues and cells) were then washed three times in PBS and then infiltrated with 1.15 M sucrose and 10% polyvinylpyrrolidone (PVP K15, Fluka, Buchs, Switzerland) in PBS for approximately 2 days at 5°C. Further ultrathin cryosectioning were carried out with a Reichert Jung FC 4E cryoultramicrotome according to Tokuyasu (1973, 1989).

Antibodies

The primary monoclonal antibodies (mouse) (mAb) used were SJ1D1 (anti-CD13; Dianova, Hamburg, Germany), Leu-M7 (anti-CD13, Becton Dickinson, Heidelberg, Germany), Ta1 (anti-CD26, Coulter, Krefeld, Germany) and BA5 (anti-CD26, Dianova, Hamburg, Germany). Polyclonal rabbit antiserum against human DPP IV (pR-DPP IV) was used to label the renal carcinoma cell line Caki-2. This serum was prepared in the laboratory of the Department of Physiological Chemistry of the University of Halle-Wittenberg. The specificity was proved by 2-dimensional immunoelectrophoresis and by Western blot described by Kettmann *et al.* (1992). The secondary antibodies (goat-anti-mouse IgG, GAM, and goat-anti-rabbit IgG, GAR), obtained from Aurion Immuno Gold (Wageningen, The Netherlands), were labelled with 6 nm, 10 nm or 15 nm gold particles (see figure legends). Optimal dilutions for use in immunolabelling were established in preliminary experiments.

Immunolabelling

The cryosections were transferred to formvar-coated nickel grids (Bio Cell Plano, Marburg, Germany), washed in PBS, afterwards in PBS containing 50 mM glycine and finally in PBS containing 0.5% albumin (Sigma, St. Louis, USA) and 0.2% gelatine (Sigma, St. Louis, USA). The sections were incubated in a moist chamber with the specific primary antiserum at 5°C overnight. The reaction with gold-labelled secondary antibodies was carried out for 1 h at room temperature. After washing in PBS and tri-distilled water, the sections were embedded in 1.1% tylose (Fluka, Buchs, Switzerland) containing 0.5% uranyl acetate. Replacing the specific primary serum with a non-specific antiserum was used as control.

At double labelling technique, CD13 and CD26 were successively labelled with antibodies raised in the same species according to Geuze *et al.* (1981). The first antigen was marked according to the standard labelling procedure with GAM 6 nm gold. The free binding sites of the primary antibody were saturated by incubation with unlabelled 0.01% goat-anti-mouse IgG (Immunpräp., Berlin) in PBG for 15 min. The other antigen was labelled with a specific antibody and GAM-conjugated gold particles of different diameter (15 nm, Aurion Immuno Gold, Wageningen, The Netherlands) as used for the first incubation. To control successfully double labelling, one of the two specific antisera was replaced by a non-specific immunserum. Additionally, the labelling sequence of CD13 and CD26 was changed. Furthermore, the double labelling results were compared with the corresponding usually single labelling. Each specimen was examined with a transmission electron microscope Zeiss EM 902 A.

RESULTS

Using the above-mentioned cryo-technique, APN and DPP IV were labelled on the external cell surfaces in renal tissues, renal cell carcinoma, cultured renal parenchymal cells and cultured renal carcinoma cells. All electron microscopic images of the cryosections showed good preservation of APN- and DPP IV-antigenicity as well as ultrastructural morphology. The optimal preservation of the plasma membranes was particularly obvious. Both cell surface peptidases were detected on the luminal side of the brush border membranes of human kidney proximal tubular cells (Fig. 1 a, b). A prominent, regular

and diffuse labelling was found on this tissue. The labelling density of APN/CD13 appeared to be higher than that of DPP IV/CD26. The double labelling experiments (Fig. 1c, d) showed a close spatial relationship between the two peptidases. Control experiments showed identical results (data not shown). As in single labelling, labelling-density for CD13 appeared to be higher than that for CD26.

On cultured renal parenchymal cells (Fig. 2), an abundant and regular expression of CD13 was detected. In comparison with renal proximal tubular cells in the tissue section, a stronger and clustered expression of CD13 was noticeable. CD26 was not found on these cells.

In renal cell cancer (mixed clear cell/chromophilic, poorly differentiated), both enzymes were labelled on the external surfaces of the brush border membranes (Fig. 3a, b). Like in benign renal tissue, we found a diffuse ultrastructural expression pattern of CD13 and CD26. But the labelling density of CD26 appeared to be higher than that of CD13. These results were almost identical with immunogold-labelling of other renal cell cancer specimens (clear cell type, moderately differentiated) (data not shown).

On the external cell surface of the renal carcinoma cells Caki-1 (Fig. 4a with CD13-labelling) and Caki-2 (Fig. 4b with DPP IV-labelling), only one of the two peptidases could be observed. These observations were confirmed by flowcytometric analyses (data not shown). In contrast to the CD13-labelling of the renal tissue cells (Fig. 1a, c, d) and the cultured renal parenchymal cells (Fig. 2), we found non-homogeneous and lower labelling in Caki-1 cells. CD13 seemed to be present in the form of clusters. When the DPP IV-expression is compared

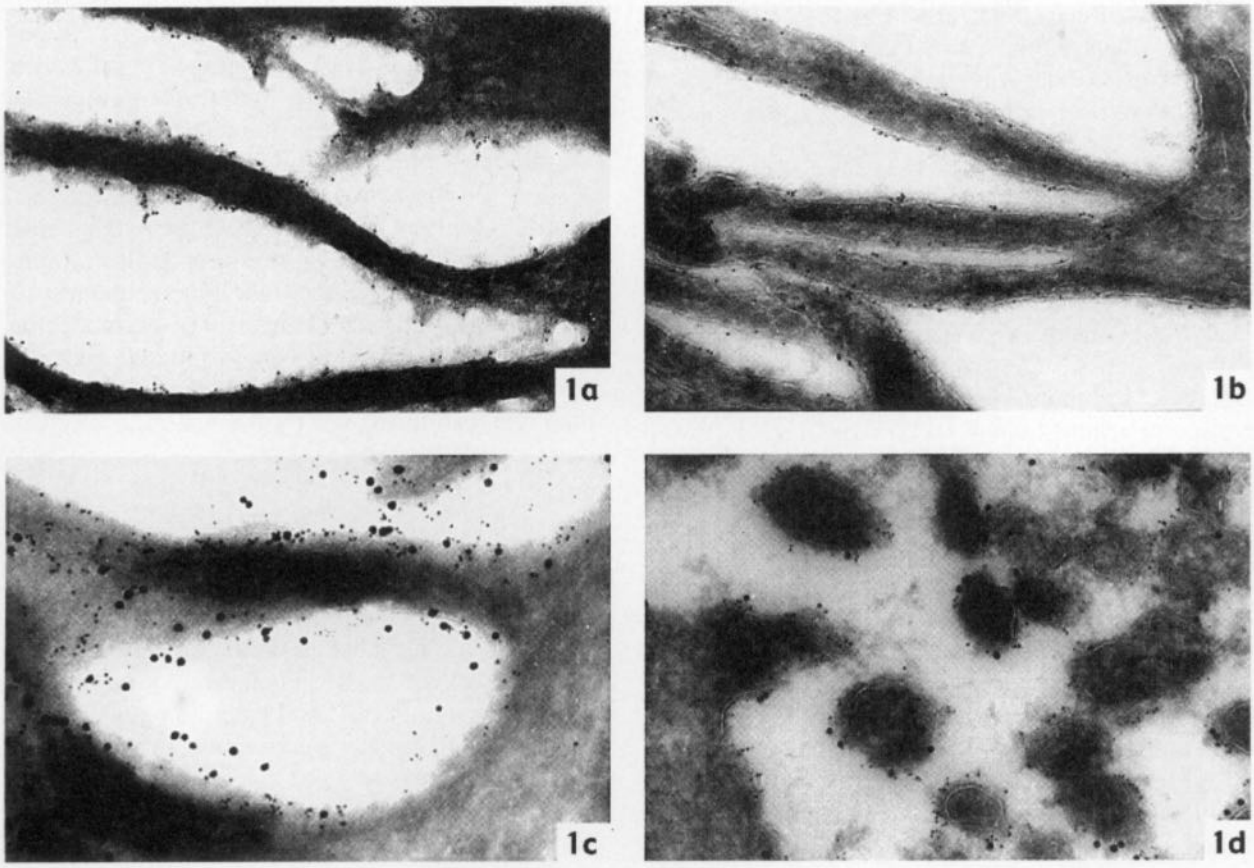

Fig. 1 - Immunolabelling of ultrathin cryosections of brush borders from human kidney proximal tubular cells. a) single labelling with CD13-mAB SJ1D1/GAM-6 nm gold. x80 000. b) single labelling with CD26-mAB BA5/GAM-6 nm gold. x80 000. c) successive double labelling with CD26-mAB BA5/GAM-6 nm gold and CD13-mAB SJ1D1/GAM-15 nm gold. x80 000. d) successive double labelling with CD13-mAB SJ1D1/GAM-6 nm gold and CD26-mAB BA5/GAM-15 nm gold. x80 000.

Fig. 2 - Single immunolabelling of ultrathin cryosection of human cultured renal parenchyma cell with CD13-mAB Leu M 7/GAM-6 nm gold. x80 000.

with renal proximal tubular cells in tissue sections, a reduced expression is observed. The expression pattern of CD26 on Caki-2 cells (Fig. 4b) showed also a discrete clustered distribution.

Control experiments in single and double labelling with non-specific antiserum did not show non-specific labelling (data not shown).

DISCUSSION

Any manipulation of the tissue during its processing can result in modifications of tissue components, leading to problems with the immunoelectron microscopic detection of antigens. Since a general procedure cannot be recommended as the best approach for immunocytochemistry, conditions for optimal labelling have to be worked out for each class of antigens and binding sites (Bendayan *et al.* 1987). Because of their exposed position on the external cell surface and their extreme glycosylation, it is difficult to localize ectoenzymes such as APN/CD13 and DPP IV/CD26 with conventional immuno-ultrastructural methods (Kettmann *et al.* 1992). Using cryo-ultramicrotomy according to Tokuyasu (1973, 1989) in combination with indirect immunogold labelling, CD13 and CD26 were detectable on the external cell surfaces in renal tissues, renal cell carcinoma, cultured renal parenchymal cells (only CD13) and cultured renal carcinoma cells. This electron microscopic technique ensures an excellent compromise between maintenance of ultrastructure and antigenicity, and has been proven to be a powerful method for the immunoelectron microscopic labelling of both cell surface and intracellular antigens and their clear assignment to ultra-

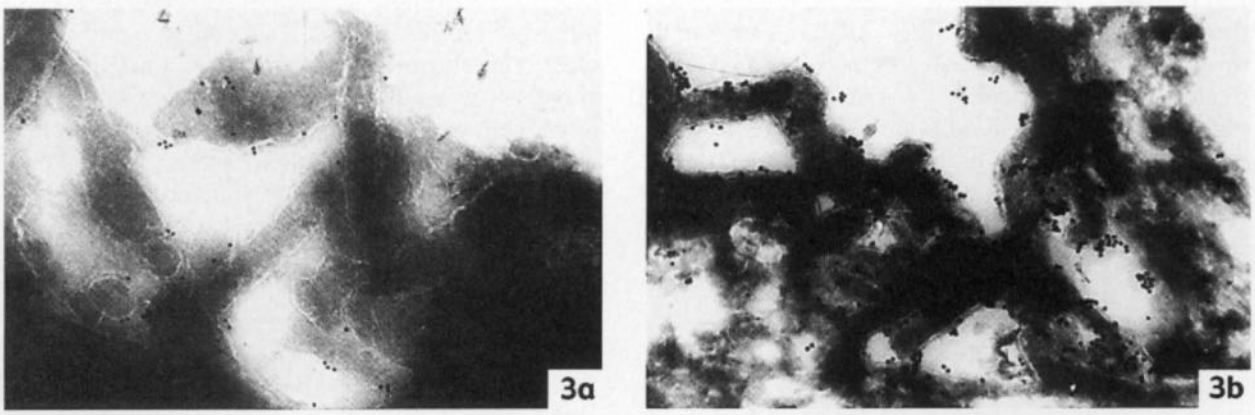

Fig. 3 - Single immunolabelling of ultrathin cryosection of human renal cancer (mixed clear cell/chromophilic). a) with CD13-mAB Leu-M7/GAM-10 nm gold. x80 000. b) with CD26-mAB Ta1/GAM-10 nm gold. x80 000.

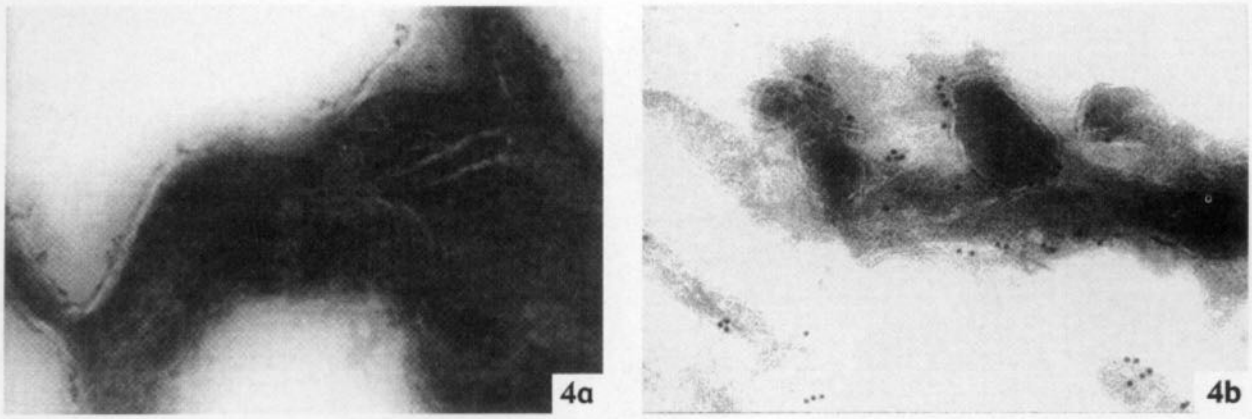

Fig. 4 - Single immunolabelling of ultrathin cryosections of human cultured renal carcinoma cells. a) Caki 1 labelled with CD13-mAB Leu M 7/GAM-6 nm gold. x130 000. b) Caki 2 labelled with pR-DPP IV/GAR-10 nm gold. x130 000.

structural compartments. Especially the correct preservation of the plasma membranes (Mizuno 1993) is responsible for the precise localization of CD13 and CD26 on various cell types.

Both membrane peptidases were abundant, regular and diffusely labelled on the external cell surface of human kidney proximal tubular cells. The expression pattern of CD13 and CD26 in single labelling was confirmed by the double labelling results. As in single labelling, labelling density of CD13 appeared to be higher than that of CD26. These observations agree with biochemical data in the literature (Kenny and Maroux 1982, McDonald and Barrett 1986). As expected, the double labelling experiments showed a close spatial relationship between the two peptidases. In this way, this successive technique is suitable for double labelling of closely related surface antigens.

The immunolabelling of CD13 on cultured renal parenchymal cells showed a stronger expression then in cells *in vivo*, but CD26 could not be found. We cannot explain this difference between the CD26 expression on these cells *in vivo* and *in vitro*. Since identical preparation conditions and antibodies were employed, methodical problems are improbable.

In renal cell cancer (mixed clear cell/chromophilic, poorly differentiated and clear cell type, moderately differentiated), CD13 and CD26 were labelled as in benign renal tissue, but labelling density of CD26 appeared a little stronger than that of CD13. In this way, CD26 appears overexpressed. On the renal carcinoma cells Caki-1 and Caki-2, in each case only one of the two peptidases could be found. In comparison with the renal proximal tubular cells, renal carcinoma cells and cultured renal parenchymal cells, CD13 was present both non-homogeneously and with lower labelling in Caki-1, where the enzyme appeared to form clusters. When CD26 on the cultured renal carcinoma cells Caki-2 is compared with renal proximal tubular cells and renal carcinoma cells in tissue sections, a reduced expression is observed. There was also a slightly different clustered expression. These entire findings match published results to a large extent.

Using PCR analysis, Kehlen *et al.* (1998) described comparable values of APN and DPP IV mRNA in non-commercially cell lines of renal tubular epithelium and renal carcinoma cells. Otherwise, commercially renal carcinoma cell lines had down-regulated (DPP IV/CD26 in Caki-1) or even lost (APN/CD13 in Caki-2) single membrane peptidases. These findings could be confirmed by immunofluorescence analysis (Kehlen *et al.* 1998). Göhring *et al.* (1998) described a strongly staining of APN and DPP IV in renal cell cancer (both in clear cell type and mixed clear cell/chromophilic type) by immunohistochemistry as well as enzyme-histochemistry. But in renal cell cancer of another histology (chromophobe, mixed clear cell/chomophilic/chomophobe, oncocytoma, mixed chomophilic/oncocytoma), a distinct lower APN and DPP IV expression could be proved (Göhring *et al.* 1998). We confirm the presence of APN and a high amount of DPP IV on renal cancers of the clear cell type as already described in the literature (Ebert *et al.* 1990, Göhring *et al.* 1998, Riemann *et al.* 1995). Moderately and well-differentiated renal carcinomas, but not anaplastic samples, expressed brush border antigens, among them

APN and DPP IV (Holthöfer *et al.* 1983). This may indicate a proximal tubular origin of these tumours, or an arrest of cellular differentiation of carcinoma cells at a stage allowing the expression of brush border antigens (Holthöfer *et al.* 1983). Because of neoplastic cell change or cell passaging, a lack of, or down-regulation of cell surface peptidases can occur. The normal co-expression of the proximal tubular specific antigens can be totally disturbed in renal carcinomas (Holthöfer *et al.* 1983). A lack of one or more antigens is a uniform feature of the malignancies irrespective of the presence of proximal tubular antigens (Holthöfer *et al.* 1983). Alterations in the carbohydrate composition of cells during malignant change have been studied on tissue sections (Nicolson and Poste 1976). Loss of expression of proximal tubule antigens over time in tissue culture is relatively rare (Ebert *et al.* 1990). Approximately twenty per cent of the renal cancer cell lines demonstrated partial or complete loss of its antigen when assayed at late passage (Ebert *et al.* 1990). Kehlen *et al.* (1998) described decreased values of mRNA for several membrane peptidases (among them CD13 and CD26) after passaging of cells. Renal cell cancer and the corresponding cell lines express proximal tubular differentiation antigens, although not uncommonly they fail to express the full complement of these antigens (Ebert *et al.* 1990). The occurrence of the peptidases depends mainly on the grade of the differentiation.

Possibly dependent on cultivation conditions, passaging or neoplastic cell change, the renal parenchymal- and renal carcinoma cell line Caki-1 and Caki-2 lose their double expression of CD13 and CD26. The clustered and reduced expression of APN in cultured renal carcinoma cells Caki 1 may be due to a changed surface structure caused by the neoplastic cell change. Compared with the renal proximal tubular cells in the tissue, the cultured renal parenchymal cells have also other cell surface characteristics. Certain cultivation conditions, or else the loss of the integrated cell system, may also result in a reduced labelling density of the APN. Therefore, in this case, conclusions of cell line characteristics from tissue cells are only possible to a limited extent. These small changes on the cell surfaces can only be detected by electron-microscopic methods.

The use of cryo-ultramicrotomy in combination with indirect immunolabelling of several cell surface antigens offer an approach for obtaining additional information on changes characterizing malignant transformation in vivo. The precise role of membrane peptidases in tumour growth and metastasis still needs to be defined. However, a deeper understanding of the many pathways regulating their expression will be necessary for a better comprehension of tumour cell growth and metastasis, but also for the development of therapeutic approaches. Further investigations, especially labelling studies on other neoplastic tissues and cells, will be necessary in order to explain the role of APN and DPP IV in malignancies.

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