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Expression patterns of the ectopeptidases aminopeptidase N/CD13 and dipeptidyl peptidase IV/CD26: immunoultrastructural topographic localization on different types of cultured cells

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Summary

Aminopeptidase N/CD13 and dipeptidyl peptidase IV/CD26 are widespread membrane-bound peptidases involved in fundamental biological processes. Using cryo-ultramicrotomy of cultured cells followed by indirect immunogold labelling, both enzymes appeared to be strongly and regularly labelled on the cell surfaces of human synovial fibroblasts, T-lymphocytes and colon carcinoma cells Caco-2. In the cytoplasm of the synovial fibroblasts gold-labelled vesicle-like structures were found, which we consider to be potential transport vesicles. An abundant and regular expression of CD13 was detected on cultured renal parenchymal cells. On the renal carcinoma cell line Caki-1 cells we found a low, non-homogeneous and clustered CD13-labelling. On cultured renal parenchymal cells and the Caki-1 cells CD26 could not be observed. The expression pattern of CD26 on renal carcinoma cell line Caki-2 cells showed also a slightly clustered distribution. A low density CD26-labelling was present on the squamous cell carcinoma cell line UM-SCC-22B. CD13 was absent in Caki-2 and UM-SCC-22B cells. The presence of both enzymes on the cultured cells enables their ultrastructural investigation under different growth conditions and their involvement in cell-cell interactions. For this purpose, however, further investigations are necessary.

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Key words: aminopeptidase N – CD13 – dipeptidyl peptidase IV – CD26 – cell surface antigens – immunoelectron microscopy – cryo-ultramicrotomy – immunogold labelling – cultured cells

Introduction

Aminopeptidase N (APN, CD13, EC 3.4.11.2) and dipeptidyl peptidase IV (DPP IV, CD26, EC 3.4.14.5) represent membrane-bound exopeptidases. They are integral, asymmetrically orientated proteins 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 aminopeptidase N is identical with the CD13 antigen (Look et al., 1989), while dipeptidyl peptidase IV corresponds with CD26 (Ulmer et al., 1990).

Both enzymes are well-characterized surface molecules expressed in a variety of cells and tissues. The function of the two peptidases varies depending on their location. The brush border membranes of renal tubular cells and small intestinal enterocytes contain an abundance of APN and DPP IV, which take part in the luminal hydrolysis of oligopeptides (McDonald and Barrett, 1986). In alveolar epithelial cells and enterocytes APN serves as a receptor for coronaviruses (Delmas et al., 1992). In several tissues APN and DPP IV modify the effects of bioactive peptides like enkephalins (APN), substance P and casomorphins (DPP IV) (De la Baume et al., 1983; Gros et al., 1985; McDonald and Barrett, 1986; Stein et al., 1989; Nausch et al., 1990). In the immune system the enzymes take part in the activation and proliferation of lymphocytes (Mentlein et al., 1984; Ashmun and Look, 1990; Bathon et al., 1992). DPP IV has a coreceptor function for HIV viruses (Callebaut et al., 1993).

In recent years, the interest in cell surface peptidases has increased considerably because, among other things, several reports indicate the involvement especially of APN and DPP IV in tumour cell invasion and the formation of metastases (Saiki et al., 1993; Menrad et al., 1993; Johnson et al., 1993; Fujii et al., 1995). Nevertheless, many functions of APN and DPP IV, both under physiological and pathological conditions, are still unknown. In order to obtain further information about the role of these peptidases, it is necessary to find out their precise ultrastructural 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 cellular 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 methodological problems (Gossrau, 1993).

When using preembedding methods intracellular antigens cannot be detected because of the non-penetration of antibodies into the cells. Postembedding methods damage the antigenicity of APN and DPP IV and therefore sufficient labelling is no longer possible (Kettmann et al., 1992 b; for further details, see Stange, 1996; Stange et al., 1996).

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 and DPP IV with conventional immunoultrastructural methods (Kettmann et al., 1992 b). The application of antibodies for the detection of the two peptidases implies that the labelling has to be performed in an aqueous environment in order to ensure detectable antigenicity. This necessity requires alternative conditions for cell preparation, since the classical methods to prepare biological material for electron microscopic examination, i. e. fixation, dehydration and plastic embedding, result in a complete loss of antigenicity in most cases. Furthermore, it has been recognized that the conventional electron microscopic methods (pre- and postembedding methods) have an enormous impact on molecular interactions so that it is difficult to draw conclusions about the morphology in the native state (Boonstra et al., 1987).

Using cryo-ultramicrotomy in combination with indirect immunogold labelling, APN and DPP IV are detectable on weakly fixed tissues (Kettmann et al., 1992 a; b; Stange et al., 1996). 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).

In the present paper the same immunoelectron microscopic technique and preparation conditions, which were satisfactorily used for tissues (see Kettmann et al., 1992 a; b; Stange et al., 1996), were tested on different cultured cells (synovial fibroblasts, T-lymphocytes, renal parenchymal cells) and carcinoma cell lines (renal carcinoma cells Caki-1 and Caki-2, colon carcinoma cells Caco-2, squamous cell carcinoma cells UM-SCC-22B) with regard to the preservation of the ultrastructural morphology and especially the maintenance of APN- and DPP IV-antigenicity. In addition, we were interested in differences or characteristics in the distribution of APN and DPP IV on the cell surfaces.

In order to assess the cryo-ultramicrotomy and preparation conditions concerning the maintenance of the peptidase antigenicity, we will compare our immunoelectron microscopic results with the already published histochemical or immunological findings.

Materials and Methods

Cells. The investigated cells were human synovial fibroblasts and human synovial IL-2 stimulated T-lymphocytes (prepared from punctures of the knee joint as described by Riemann et al., 1993), the human renal tubular epithelial cell line NP 33 (pre-

pared from nephrectomy tissues as described by Riemann et al., 1995), the human renal carcinoma cell lines Caki-1 (ATCC) and Caki-2 (ATCC), the human colon carcinoma cell line Caco-2 (ATCC) and the squamous cell carcinoma cell line UM-SCC-22B (metastase of a squamous cell carcinoma of the hypopharynx; Carey, 1994; obtained from T. Carey, University of Michigan Cancer Center, USA).

Antibodies. The primary monoclonal antibodies (mAb) used were SJ1D1 and WM 15 (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). In order to increase the sensitivity, the CD13-labelling of T-lymphocytes and the colon carcinoma cell line Caco-2 was carried out with a mixture of the three mAbs SJ1Dl, WM 15 and Leu-M7. Polyclonal rabbit antiserum against human CD26 (pR-DPP IV) was used to label the renal carcinoma cell line Caki-2 and the colon carcinoma cell line Caco-2. This serum was prepared in the laboratory of the Department of Physiological Chemistry of the University of Halle. 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 12 nm gold particles.

Electron microscopy (for details; see Stange, 1996; Stange et al., 1996). After centrifugation and washing in PBS, the cells were fixed in 2% formaldehyde and 0.02% glutaraldehyde in PBS for 20 min at room temperature, 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. After washing in PBS, all fixed samples were incubated with 1.15 M sucrose and 10% polyvinylpyrrolidone (PVP K15, Fluka, Buchs, Switzerland) in PBS for approximately 2 days at 5 °C. Further processing and cryosectioning were carried out according to Tokuyasu (1973; 1989). Ultrathin cryosectioning was performed with a Reichert-Jung FC 4E cryoultramicrotome.

Immunolabelling (for details; see Stange, 1996; Stange et al., 1996). 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 tridistilled water, the sections were embedded in 1.1% tylose (Fluka, Buchs, Switzerland) containing 0.5% uranyl acetate. Controls were carried out by replacing the specific primary serum with an unspecific antiserum.

Results

The electron microscopical images of the ultrathin cryosections of the human synovial fibroblasts (Fig. 1 a, b), T-lymphocytes (Fig. 2 a, b), renal parenchymal cells (Fig. 3), renal carcinoma cells Caki-1 (Fig. 4 a) and Caki-2 (Fig. 4 b), colon carcinoma cells Caco-2 (Fig. 5 a, b) and squamous cell carcinoma cells UM-SCC-22B (Fig. 6) showed excellent preservation of the cell structures. The optimal preservation of the plasma membranes was particularly obvious. The maintenance of the CD13- and CD26-antigenicity on these cells was also very good (Figs. 1–6). Using the preparation conditions described earlier, cryo-ultramicrotomy in combination with the indirect im-

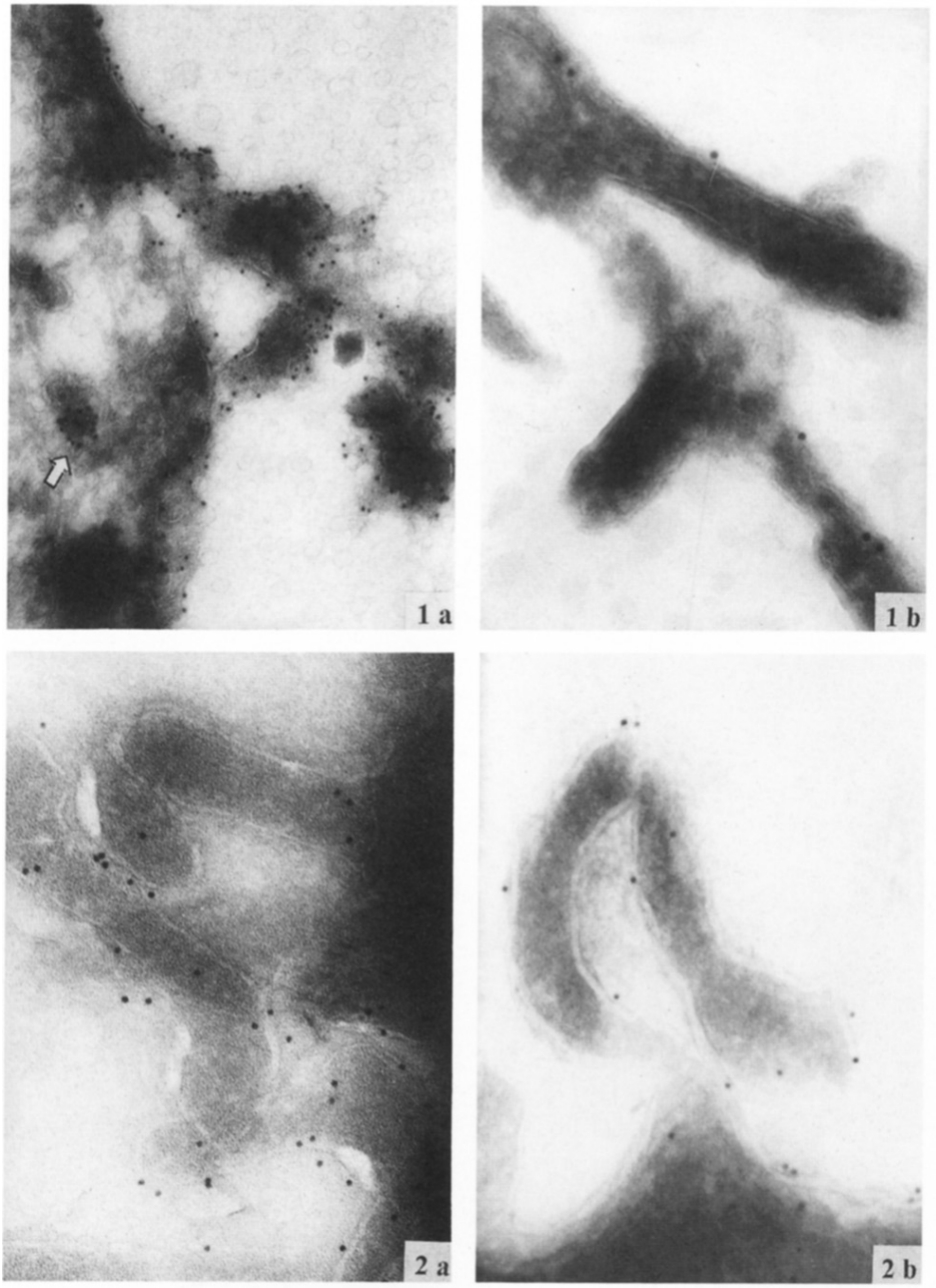
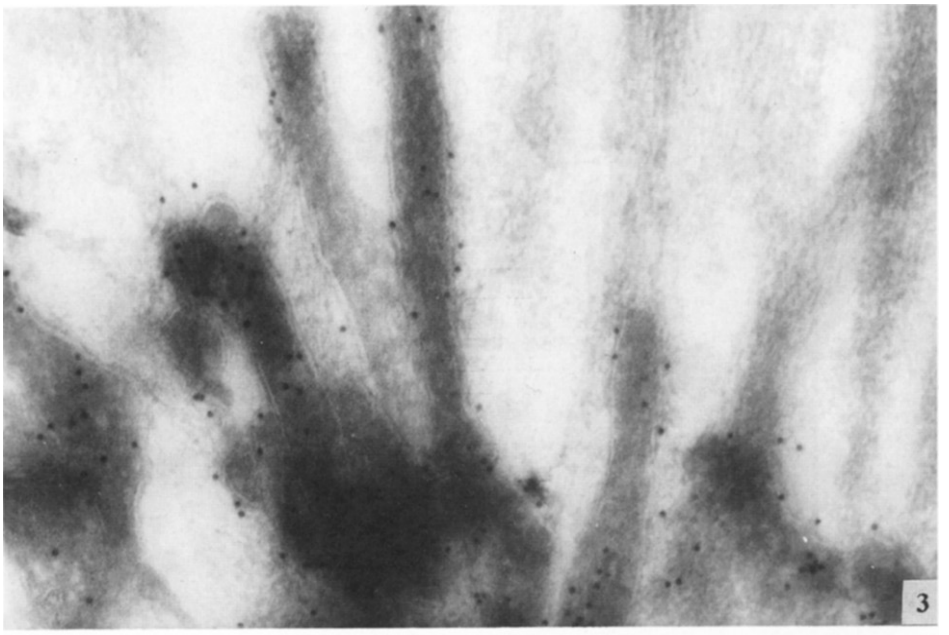
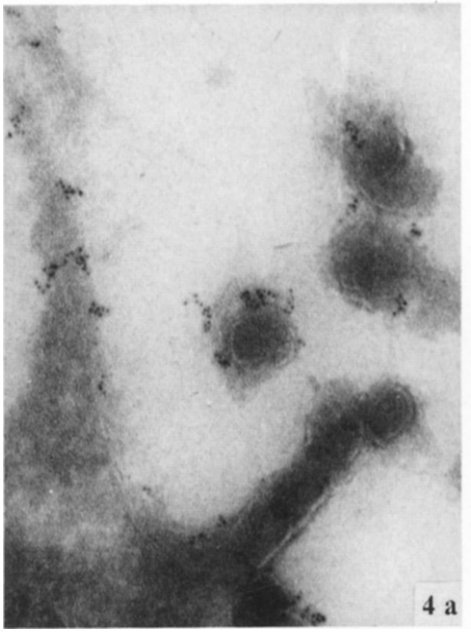


Fig. 1. Immunolabelling of ultrathin cryosections of human synovial fibroblasts. **a**. With CD13(SJ1D1)-mAb/GAM-12 nm gold; the arrow shows a gold labelled intracellular vesicle. ×110,000. **b**. With CD26(BA5)-mAb/GAM-12 nm gold. ×80,000.
Fig. 2. Immunolabelling of ultrathin cryosections of human T-lymphocytes. **a**. With a mixture of CD13-mAb's (SJ1D1, WM 15, Leu-M7)/GAM-12 nm gold. **b**. With CD26(BA5)-mAb/GAM-12 nm gold. ×80,000.





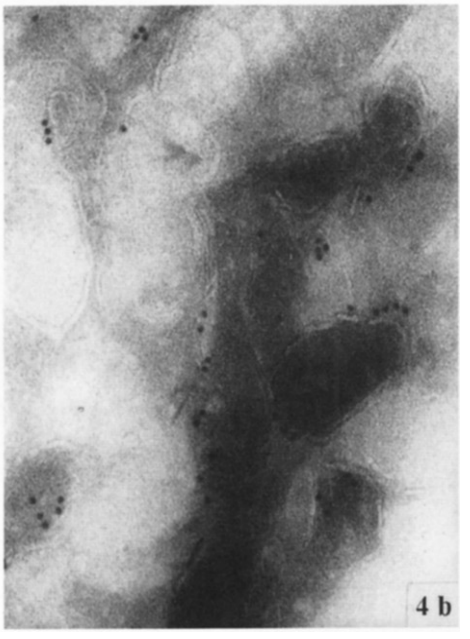


Fig. 3. Immunolabelling of an ultrathin cryosection of a human cultured renal parenchyma cell with CD13(Leu-M7)-mAb/GAM-12 nm gold. ×80,000. Fig. 4. Immunolabelling of ultrathin cryosections of human cultured renal carcinoma cells. **a**. Caki-1 cell line labelled with CD13(SJ1D1)-mAb/GAM-6 nm gold. **b**. Caki-2 cell line labelled with pR-DPP IV/GAR-10 nm gold. ×80,000.

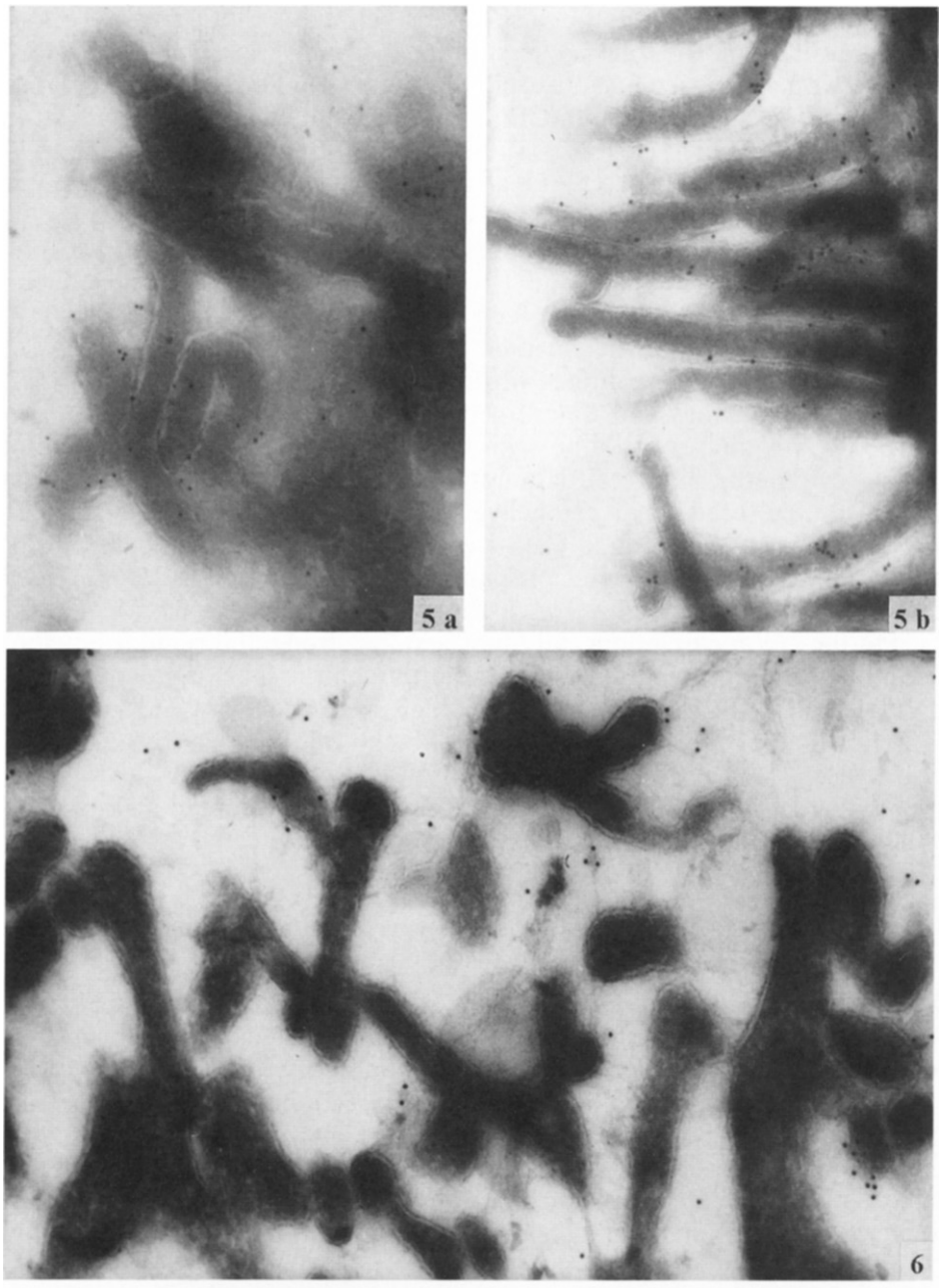


Fig. 5. Immunolabelling of ultrathin cryosections of human cultured colon carcinoma cell line Caco-2. **a**. With a mixture of CD13-mAbs (SJ1D1, WM 15, Leu-M7)/GAM-10 nm gold. **b**. With pR-DPP IV/GAR-10 nm gold. ×80,000. Fig. 6. Immunolabelling of an ultrathin cryosection of a squamous cell carcinoma cell line UM-SCC-22B with CD26(Ta1)-mAb/GAM-10 nm gold. ×80,000.

munogold labelling resulted in a specific labelling of CD13 and CD26 on the external sides of the plasma membranes. Control experiments (data not shown) with an unspecific antiserum did not reveal non-specific labelling.

With SJ1D1-mAb against CD13 the synovial fibroblasts were strongly and regularly labelled (Fig. 1 a). The labelling with Leu-M7-mAb led to identical results (data not shown). Near the cell surface in the cytoplasm immunogold-labelled vesicles were visible (Fig. 1 a). In contrast to the strong staining of CD13, a distinct lower expression of CD26 was found (Fig. 1 b). On the ultrathin cryosections of the T-lymphocytes an intense homogeneous labelling of both peptidases was seen (Fig. 2 a, b). An abundant and regular expression of CD13 was detected on the renal parenchymal cells (Fig. 3).

On the external cell surface of the renal carcinoma cells Caki-1 (Fig. 4 a with CD13-labelling) and Caki-2 (Fig. 4 b 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 cultured renal parenchymal cells (Fig. 3) we found non-homogeneous and lower labelling in Caki-1 cells. CD13 seemed to be present in the form of clusters. The expression pattern of CD26 on Caki-2 cells (Fig. 4 b) showed also a discrete clustered distribution.

On the colon carcinoma cell line Caco-2 a strong and regular labelling of both membrane enzymes was present, although the labelling density of DPP IV (with pR-DPP IV) appeared to be higher than that of CD 13 (Fig. 5 a, b). The labelling of CD26 with the Ta1-mAb showed identical results (data not shown).

A low density of CD26-labelling appeared on the squamous cell carcinoma cell line UM-SCC-22B (Fig. 6). CD13 was not detected on these cells.

Discussion

The preservation of antigenicity during the electron microscopical preparation process represents the basic requirement for the subcellular localization of membrane-bound exopeptidases. In addition, the ultrastructural cell morphology must be maintained. Ultrathin cryosectioning according to Tokuyasu (1973, 1989) in combination with indirect immunogold labelling 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 ultrastructural 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 (all figures). This is particularly due to the excellent preservation of the double layers of the plasma membrane, especially when compared with immunoelectron microscopical images obtained with conventional preparation techniques.

The same preparation conditions, which were applied to tissues with satisfactory results (see Kettmann et al., 1992 a, b; Stange et al., 1996), were

used here for the preparation of various cell types. Owing to the impermeability of the cross-linked gelatine matrix for the gold probe, gelatine-embedded cells can reduce immunogold labelling, especially when they are postfixed (Van Bergen en Henegouwen, 1989), an observation which is particularly true for cell surface antigens. In our investigation, however, peptidase antigenicity was excellently preserved in spite of postfixation of the gelatine embedded cells.

In agreement with Bathon et al. (1992), we could show CD13 and CD26 on the cell surface of the synovial fibroblasts. The CD13 antigen is considerably stronger labelled than CD26. Using biochemical methods Bathon et al. (1992) described a three-to-one relation between CD13 and CD26. These results correspond to our findings. The CD13-labelled vesicles, seen near the cell surface in the cytoplasm, may be vesicles possibly transporting the membrane enzymes from the Golgi complex to the cell surface (Danielsen et al., 1987). In the Golgi complex no immunogold labelling was found. In proximal tubular cells from human kidney (DPP IV; Stange et al., 1996), proximal tubular cells from rat kidney (APN; Kettmann et al., 1992 b), rat small intestinal enterocytes (APN; Stange, 1996), pig small intestinal enterocytes (APN; Hansen et al., 1987) and in the human colon carcinoma cell line Caco-2 (DPP IV; Klumperman et al., 1991) the membrane peptidases could be labelled and have been discussed as potential transport vesicles. In the other cells no intracellular labelling was present. The intracellular labelling of the synovial fibroblasts is probably due to very strong cell surface labelling of CD13 followed by endocytotic uptake.

Our immunoultrastructural detection of CD13 and CD26 on the stimulated T-lymphocytes coincides with the biochemical findings by Mentlein et al. (1984) for the DPP IV and Riemann et al. (1993) for the APN. Using cytofluorometry Riemann et al. (1993) found also APN-expression on these IL-2 stimulated T-lymphocytes and regarded the enzyme as a new activation-associated molecule on these cells. The possible function of APN and DPP IV on the T-lymphocytes is still a matter of speculation. It is probable that both peptidases act in a concert with other ectopeptidases to inactivate or to activate signalling peptides.

The immunolabelling of CD13 on cultured renal parenchymal cells shows a lower expression than in cells in vivo (Kettmann et al., 1992 a, b; Stange, 1996; Stange et al., 1996). These results are comparable, because they were obtained with the same methods. On the renal carcinoma cells Caki-1 and Caki-2, in each case only one of the two peptidases could be found with cryo-ultramicrotomy. These results coincide with our findings obtained by fluorescence analysis. In comparison with the cultured renal parenchymal cells and renal proximal tubular cells, from tissue sections (Kettmann et al., 1992 b; Stange et al., 1996), CD13 is present both non-homogeneously and with lower labelling in Caki-1 cells, where the enzyme appears to form clusters. When CD26 on the cultured renal carcinoma cells Caki-2 is compared with renal proximal tubular cells in tissue sections (Kettmann et al., 1992 a; Stange et al., 1996), a reduced expression is observed. There is also a slightly different clustered expression, which would probably be stronger with 6 nm

instead of the applied 10 nm gold particles. Small gold particles (diameter less than 7 nm) label with the highest efficiency, because they penetrate the cryosections better leading to a higher labelling density (Van Bergen en Henegouwen and Leunissen, 1986).

Possibly the renal carcinoma cell lines Caki-1 and Caki-2 represent mutants and lost their potential for the expression of the two peptidases. The clustered and reduced expression of CD13 in cultured renal carcinoma cells Caki-1 may be caused by a changed surface structure induced by the neoplastic cell process or to the loss of the integrated cell system. These slight changes of the cell surfaces can only be detected by electron microscopical methods. Therefore, in this case conclusions from cell line characteristics to tissue cells are only possible to a limited extent. Further investigations, especially double-labelling studies with an additional plasma membrane enzyme, will be necessary in order to explain the findings in these carcinoma cells.

The strong and regular immunogold labelling of CD13 and CD26 on the colon carcinoma cell line Caco-2 (Fig. 5 a, b) are in accordance with published immunohistochemical results. The expression pattern of CD26 occurs almost invariably (Klumperman et al., 1991; Howell et al., 1993; Vachon et al., 1996) in contrast to CD13, which is present only on the surface of some cells (Howell et al., 1993; Vachon et al., 1996). In our investigations, both peptidases were homogenously detectable in all analysed cells.

Unlike CD13, CD26 is present on the cell surface of the squamous cell carcinoma cell line UM-SCC-22B. CD26 has also been found in squamous cell carcinomas of the lung (Sedo et al., 1991). Moehrle et al. (1995) did not find CD13 in the tumour tissue of squamous cell carcinomas and CD26 in poorly differentiated squamous cell carcinomas of the skin. However, CD26 was detectable on differentiated squamous cell carcinomas of this organ. These results agree with our findings, which are also supported by our immunohistochemical investigations on squamous cell carcinomas of the head and neck (unpublished observations).

Summarising, we have investigated the plasma membrane peptidases CD13 and CD26 on various types of cultured cells. Using cryo-ultramicrotomy of weakly fixed and gelatine-embedded cells in combination with an indirect immunogold labelling, CD13 and CD26 are excellently visible on plasma membranes. In one case intracellular labelling of vesicle-like structures was achieved. Our immunoelectron microscopic findings coincide with results from the literature. Because of the detection of both enzymes on cultured cells, their ultrastructural investigation under different growth conditions and of their potential role in cell-cell interactions is possible, the details of which have to clarified by future investigations.

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