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Immunohistochemical localization of dipeptidyl aminopeptidase IV in thyroid papillary carcinoma

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Summary. The localization of dipeptidyl aminopeptidase IV expressed aberrantly in thyroid carcinoma was studied by immunoelectron microscopy using a monoclonal antibody to the enzyme with special reference to enzyme-histochemical staining of the enzyme. Five thyroid papillary carcinomas were investigated including two lymph-node metastases. All cases showed the dense immunoreaction product on the apical membrane and only traces of the product on lateral membranes, endoplasmic reticulum and nuclear membranes. In one case only, the dense product was observed on basal tubular structures. Analysis, using immunogold labelling on pre-embedded cryosections, revealed that dipeptidyl aminopeptidase IV was localized on the luminal surface of cancer cells. Two different distribution patterns of dipeptidyl aminopeptidase IV activity staining, diffuse and apical patterns, reported previously were thought to be due to different amounts of dipeptidyl aminopeptidase IV in the cytoplasm of cancer cells. This enzyme-histochemical staining method is useful for pathological diagnosis of thyroid tumours and can be applied to clinical materials. The enzyme localization is revealed by the staining pattern.

Keywords: DAP IV, thyroid papillary carcinoma, monoclonal antibody, immunohistochemical localization.

Dipeptidyl aminopeptidase IV (DAP IV) (EC 3.4.14.5) is a membrane-bound enzyme discovered by Hopsu-Havu and Glenner (1966). Its distribution in normal tissues has been extensively studied by means of both enzyme-histochemical and immunohistochemical procedures. In the thyroid gland, however, DAP IV is found in the capillary endothelia but not in the follicular epithelial cells (Gossrau 1979).

We have recently reported that thyroid carcinoma expresses aberrant DAP IV activity and that enzyme-histochemical staining of DAP IV is very useful for pathlogical diagnosis of thyroid tumours (Kotani *et al.* 1991; Aratake *et al.* 1991). We observed two staining patterns in tumours, namely, diffuse and apical patterns. A possible explanation of the two different staining patterns was thought to be differences in DAP IV subcellu-

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lar localization in different individuals. To confirm this hypothesis, we attempted to clarify the localization of DAP IV in thyroid carcinoma by immunoelectron microscopy.

In this paper, we report results from five thyroid papillary carcinomas including two lymph-node metastases. Results partially support our hypothesis concerning the relationship between the immunohistochemical localization of DAP IV and the enzyme-histochemical staining patterns.

Materials and methods

Thyroid tissues

Thyroid tissue obtained immediately after surgical removal was cut into three pieces. Part was fixed with 15% formalin and processed for histopathological analysis. Another part was embedded in Tissue Tek II OCT (OCT-compound, Miles Lab., Naperville, In) followed by freezing for enzyme-histochemistry. For immunoelectron microscopic study the third part was fixed with periodate–lysine–paraformaldehyde, embedded in Tissue Tek II OCT, and then frozen in liquid nitrogen (Nakagawa *et al.* 1985). The final diagnosis was based on pathological findings, and tumours were classified according to the World Health Organization classification of thyroid tumours (Hedinger *et al.* 1988).

Enzyme-histochemistry for DAP IV

DAP IV activity was revealed histochemically by the method described previously (Kotani *et al.* 1991). Briefly, 6–8-μm frozen sections were fixed with acetone for 60 s, incubated with the reaction mixture in a moist chamber at room temperature for 30 min and counterstained with Carazzi's haematoxylin solution for 5 min.

Monoclonal antibody to DAP IV

Human placental DAP IV was partially purified by a slightly modified method of Wolf *et al.* (1989). The final preparation had a specific activity of 20.1 U/mg protein when measured by a chromogenic method (Nagatsu *et al.* 1976). In 7.5% cross-linked sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reduced conditions, the preparation showed bands at 105 and 80 kDa. The densitometric analysis revealed that 105 kDa band comprised 65%.

Spleen cells of BALB/c mice immunized with partially purified DAP IV were isolated and fused with P3-X63-Ag8-U1 (P3U1) mouse myeloma cells with the aid of 50% polyethylene glycol 1500 (Nakagawa *et al.* 1985). After the HAT selection for 13 days, the resultant hybridoma cells were examined for their ability to produce antibodies to DAP IV by the enzyme-linked immunosorbent assay (ELISA). Positive wells for antibody activity were subcloned twice. Eighteen clones were re-examined for their antibody production and trapping of DAP IV activity. One of the 18 clones (44.3) showed the highest and most specific binding activity toward the DAP IV and was further analysed for its specificity using IgG purified from ascitic fluids. In the adsorption test with an immunoaffinity column of Sepharose 4B (Pharmacia Biotech, Piscataway, NJ) coupled with 44.3 IgG, human placental microsome solubilized with 1% Triton X-100 lost its DAP IV activity (specific activity of 543 mU/mg protein to less than 0.1 mU/mg protein). The eluate had a higher specific activity of 41.2 U/mg protein and showed a 106 kDa band which recorded 98% in a densitometer scanning. Isotype, pI value and molecular weights of heavy and light chains of the monoclonal antibody 44.3 IgG were also determined to be IgG1, 6.4, 50 kDa and 26.5 kDa, respectively. $F(ab')_2$ of 44.3 was prepared and conjugated with horseradish peroxidase (HRP) to get Fab'-HRP by the method described previously (Nakagawa *et al.* 1985).

Immunoperoxidase staining

Cryosections (6–8 μm thick) were treated

Table 1. Cases studied in this report

Case		Age	Sex	Pathological diagnosis	DAP IV		Electron microscopy findings			
					Activity	Staining %	Am	MS	LM	ERNM
1	KA	60	F	Papillary carcinoma (moderately differentiated)	+++	70*	+++**	±	±	±
2	KO	58	F	Papillary carcinoma (well differentiated)	+++	>70	+++	±	±	±
3	YN	60	M	Lymphnode metastasis of papillary carcinoma (well differentiated)	+++	70	+++	±	±	±
4	KM	53	F	Lymphnode metastasis of papillary carcinoma (moderately—well differentiated)	+++	70	+++	++	±	±
5	TT	64	F	Papillary carcinoma (moderately differentiated)	++	50	++	±	±	±
6	KM	34	F	Adenomatous goitre	−	<10	±	−	−	−
7	KT	41	F	Adenomatous goitre	−	<10	−	−	−	−
8	MF	48	F	Adenomatous goitre	++	50 ++	−	−	−	
9	KY	48	F	Follicular adenoma	−	<10	−	−	−	−
10	EG	31	M	Graves' disease	−	<10	−	−	−	−
11	MT	18	F	Graves' disease	−	<10	−	−	−	−
12	TN	63	F	Chronic thyroiditis	−	<10	−	−	−	−
13	EK	57	F	Chronic thyroiditis	−	<10	−	−	−	−

* Staining was evaluated by staining intensity (−, negative; +, weakly staining; ++, moderately staining; +++, strongly staining) and number of staining cells (Kotani *et al.* 1991).

** Deposit of HRP-reaction products was estimated as follows: −, no products; ±, trace-amount of products; +, small amount of products; ++, large amount of products.

NT, Not tested.

AM, Apical membrane; MS, microtubular structure; LM, lateral membrane; ERNM, endoplasmic reticulum and nuclear membrane.

with 44.3 Fab'-HRP according to the procedure described previously (Nakagawa *et al.* 1985).

Endogenous peroxidase activity was completely inhibited by 0.1 M sodium azide in most but not all cases. Specific binding of Fab'-HRP was absorbed by preincubation of 44.3 Fab'-HRP (0.25 μg/ml) with DAP IV (80 μg/ml) overnight at 4°C (data not shown).

Immunogold staining

In order to biotinylate 44.3 IgG, the IgG (0.6 mg) was incubated with 0.06 mg of *N*-hydroxysuccinimidobiotin (Pierce Chemical Co., Rockford, Il) in 1 ml of 0.1 M $NaHCO_3$ overnight at 4°C. After incubation, free *N*-hydroxysuccinimidobiotin was removed by dialysis against phosphate buffered saline (PBS).

Antistreptavidin–colloidal gold was prepared as follows. Preparation of colloidal gold (about 10 nm) by the method of Slot and Gueze (1985) was conjugated with anti-streptavidin (Vector, Burlingame, Ca) by the procedure of De Mey (1986).

Cryosections (15 μm thick), treated with normal BALB/c serum, were serially incubated with 1 μg/ml biotinylated 44.3 IgG in 1% bovine serum albumin (BSA)–PBS overnight at 4°C, 2 μg/ml streptavidin (Sigma Chemical Company, St Louis, Mo.) in BSA–PBS for 3 min at room temperature, and antistreptavidin–colloidal gold diluted with PBS containing 0.02% Carbowax 20M (Union Carbide, NY) for 30 min at room temperature.

Non-specific binding of streptavidin and antistreptavidin–colloidal gold to the sections were not detected (data not shown).

Electron microscopy

Sections stained with immunoperoxidase and immunogold procedures were post-fixed with 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4) for 30 min, dehydrated with a graded series of ethanols, and then embedded in Epon 812. Ultrathin sections were cut with an LKB Ultrotome, briefly counterstained with uranyl acetate and lead citrate, and examined with JEM 1200 EX electron microscope.

Results

The DAP IV localizations in five thyroid papillary carcinomas, including two lymph-node metastases, were revealed by both enzyme-histochemistry and immunohistochemistry (Table 1). Enzyme-histochemically, DAP IV was demonstrated to be very active in four carcinomas (cases 1–4), while in the fifth case (case 5) it was only moderately active. In four cases (cases 1–3, 5) the reaction products of DAP IV were localized only on the apical membranes of cancer cells (apical pattern, Fig. 1b), whereas in case 4, the reaction products of DAP IV were present in the cytoplasm as well as on the apical plasma membranes of the cancer cells (diffuse pattern, Fig. 1a). Other thyroids with various disorders showed no activity for DAP IV except for case 8 which had an apical staining pattern.

By immunoelectron microscopy, four carcinomas (cases 1–4) had the dense immunoperoxidase reaction product located on almost all apical membranes of the cancer cells (Fig. 2) but in case 5 not all the apical membranes stained. In case 4, in addition to apical membranes, basal tubular structures in the cancer cells contained the dense immunoperoxidase reaction product, which enzyme-histochemically equated to the diffuse pattern (Fig. 2). The other carcinomas showed only trace amounts of the product in these structures. Lateral membranes, endoplasmic reticulum and nuclear membranes of cancer cells also showed trace amounts of the product. Three adenomatous goitres lacked the immunoreaction product except for case 8 which had moderately intense staining of the apical membranes of the nodular part.

A further study with immunogold label-

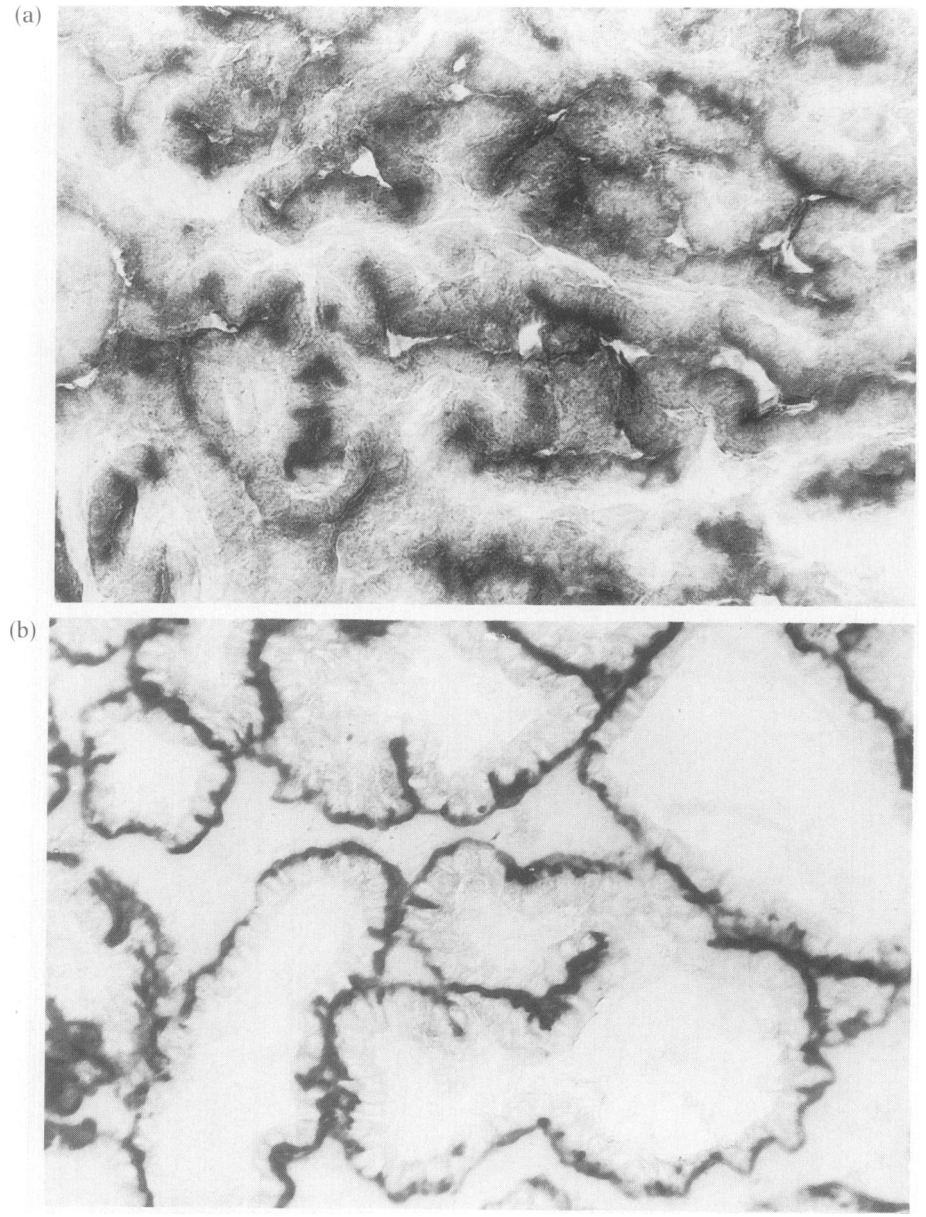

Fig. 1. DAP IV activity staining. a, In case 4, both apical membrane and inter-cellular region and cytoplasm are strongly stained. b, In case 2, only apical membrane is stained × 200.

ling demonstrated the presence of DAP IV on the luminal surface (Fig. 3).

Discussion

The aberrant expression of DAP IV activity in thyroid carcinoma is very useful for pathological diagnosis (Kotani *et al.* 1991, Aratake *et al.* 1991), although two staining patterns of DAP IV activity, diffuse and apical patterns, were observed. In order to test the hypothesis that different staining patterns of

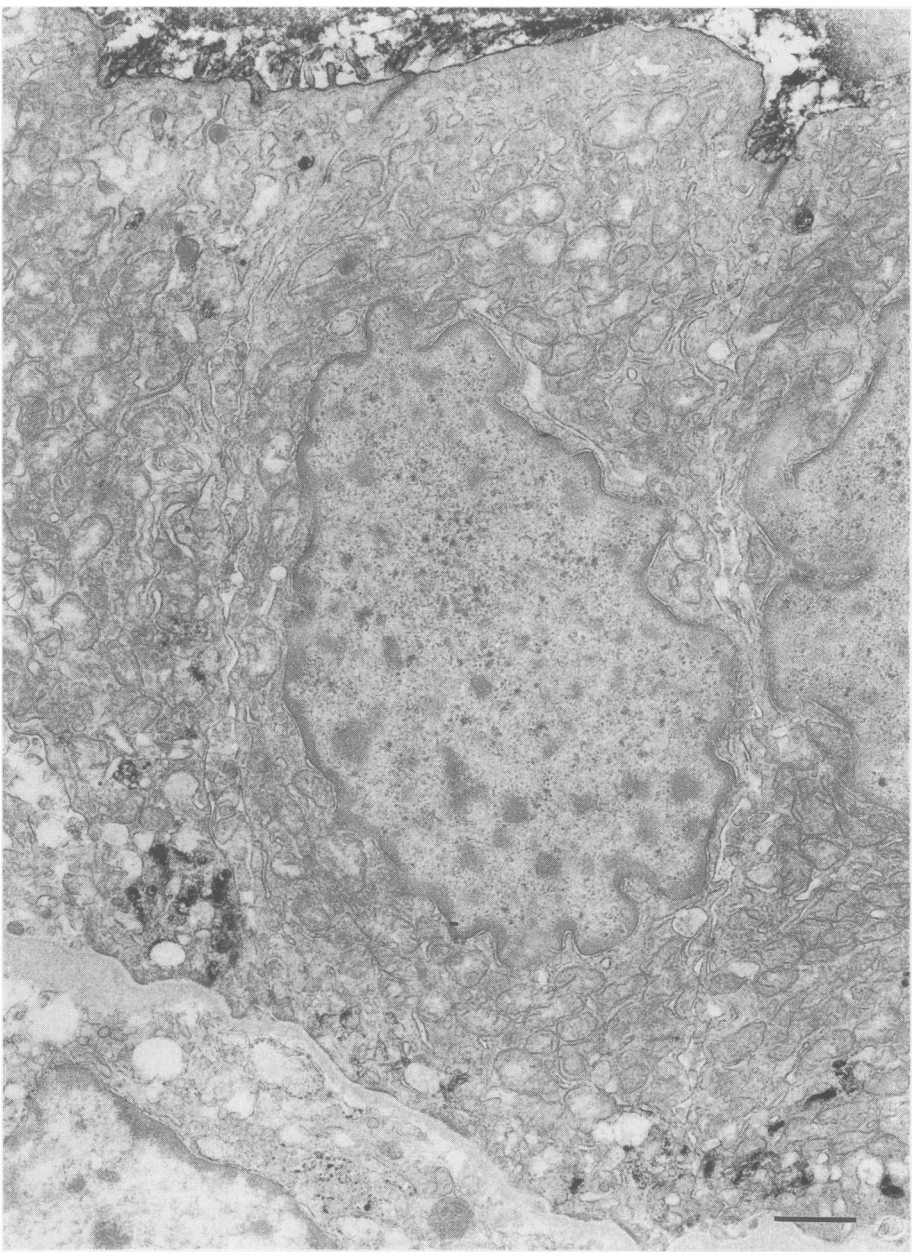

Fig. 2. Immunoelectron micrograph illustrating a direct immunoperoxidase method using a monoclonal antibody to DAP IV. Apical membrane and microvilli showed dense immunoreaction product. In this case (case 4), the product was also observed on basal tubular structures. Lateral membrane, endoplasmic reticulum, and nuclear membrane showed trace amounts of the product. Bar 1 μm.

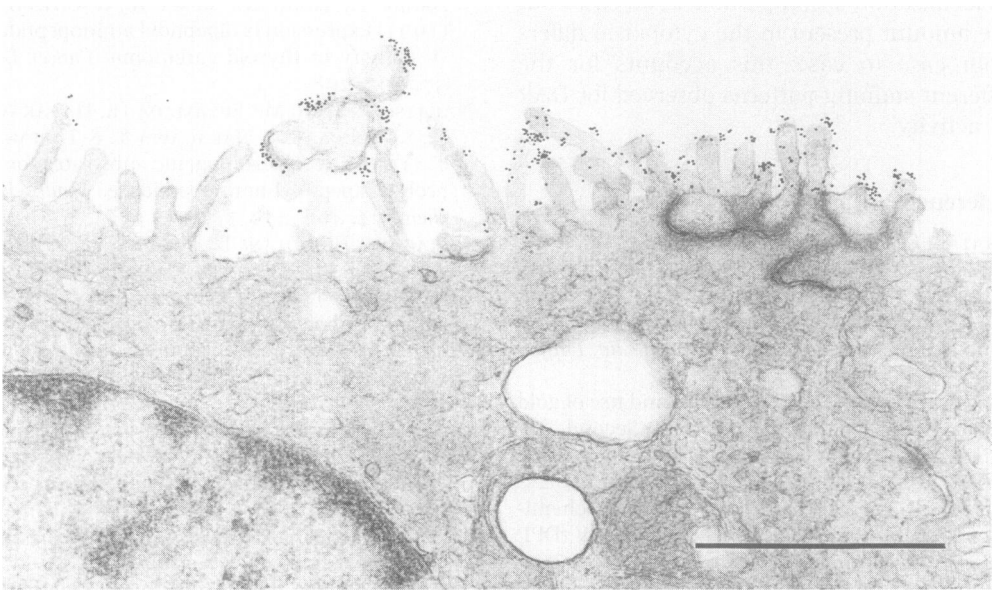

Fig. 3. Immunogold labelling of DAP IV on a pre-embedding cryosection, showing that DAP IV is localized on luminal surface of a carcinoma cell in case 1. Bar 1 μm.

DAP IV were due to different subcellular localizations of DAP IV, we investigated DAP IV localization in thyroid carcinoma by immunoelectron microscopy using a monoclonal antibody to DAP IV. Five papillary carcinomas showed almost the same localization pattern; a large amount of apical membranes and trace amounts only on lateral membranes, endoplasmic reticulum, and nuclear membranes, although one case (case 4) showed high DAP IV activity on basal tubular structures as well as on apical membranes. This case had a diffuse staining pattern for DAP IV activity by enzyme-histochemistry, as shown in Fig. 1a and Table 1. These immunocytochemical findings of DAP IV appear compatible with those of DAP IV enzyme-histochemistry. This study therefore suggests that differences in enzyme and immuno-histochemical localization of DAP IV in the thyroid cancers examined, reflect the amounts of enzyme expressed *in situ*. Cancer cells of case 4 were moderately to well differentiated and showed dense HRP reaction products on basal tubular structures. In the previous study of DAP IV activity staining, the diffuse pattern was observed more frequently in carcinomas with lower differentiation. DAP IV activity staining pattern may thus be related to the degree of cancer cell differentiation.

The immunogold labelling experiment showed that DAP IV activity was localized on the luminal side of the apical membrane (Fig. 3). Ogata *et al.* (1989) cloned rat DAP IV cDNA to analyse and deduced its primary structure. They also found that the signal sequence remains without being cleaved and works as a membrane-anchoring domain. Using their cDNA as a probe, we analysed mRNA size for human liver, kidney and thyroid carcinomas by Northern blot analysis. The mRNAs of these three organs were the same size (unpublished observation). We therefore concluded that DAP IV in thyroid carcinoma was only aberrantly expressed and did not have any molecular abnormality.

This report describing DAP IV localization in thyroid papillary carcinoma suggests that

its localization is comparable in all cases but the amount present in the cytoplasm differs from case to case; this accounts for the different staining patterns observed for DAP IV activity.

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