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Immunohistochemical localization of endothelin-1 in non-neoplastic and neoplastic adrenal gland tissue

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Abstract Endothelin (ET)-1 is a 21-amino acid peptide with potent vasopressor and vasocontrictive properties. Biochemical studies suggest that this peptide occurs in adrenal glands, where it influences steroid hormone production. However, we have found no report of the topographical distribution of this peptide. The localization of ET-1 immunoreactivity in non-neoplastic (37 cases) and neoplastic adrenal glands (48 cases) was investigated with a sensitive immunohistochemical technique applied to routinely processed tissue specimens. ET-1 immunoreactivity was regularly seen in the cortex, especially in the zona fasciculata and to a varying extent also in the other two zones, but not in the medulla. The immunoreactive material appeared in the cytoplasm mostly in the form of vacuolar structures but also as grains. Focally, the cell membrane also showed immunoreactive staining. In the zona reticularis the immunoreactivity appeared mainly as cytoplasmic grains. Most cortical adenomas displayed numerous immunoreactive cells. The immunoreactivity in the tumour tissue appeared in the same forms as in normal cortex, but the reactive products were generally fewer in number. No obvious differences in immunostaining were seen between the aldosterone- and cortisol-producing adenomas or the non-functioning ones. Three of the ten carcinomas contained immunoreactive cells, but they were few, appearing focally and the ET-1 immunoreactive structures were seen as 'dust-like' material. The difference in immunoreactivity between the benign and the malignant cortical neoplasms may be of diagnostic value. Functionally our results support a relationship between ET-1 and steroid regulation in non-neoplastic cortical tissue.

Key words Endothelin-1 · Adrenal gland
Adrenal tumour · Immunohistochemistry

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Introduction

Endothelin (ET)-1, a 21-amino acid peptide with potent vasopressor and vasocontrictive properties, was originally isolated from the supernatant of porcine endothelial cell cultures (14). Recently, three distinct human ET-related genes were identified. ET-1 differs by two and six amino acids from ET-2 and 3 respectively (5). It has been shown that ET-1 stimulates hormone secretion from various endocrine and neuroendocrine cells (atrial natriuretic peptide from rat atrial myocytes, catecholamines from bovine chromaffin cells, and vasopressin from rat hypothalamus (6). Recently it has been reported that ET-1 in vitro activates the secretion of aldosterone or cortisol and corticosterone – or all these three steroids – from zona glomerulosa of calf (1) or from this zone and from zona fasciculata of rat and human (4).

The localization of ET-1 mRNA and ET-like immunoreactivity has been demonstrated in neurones of the human brain (3), spinal cord and the dorsal root ganglia (2). Specific high-affinity binding sites for ET have been found in brain, heart, arteries, kidney, lung, intestine of rat, in brain of human, but also in other tissues by using autoradiographic technique and receptor-binding assays with ^{125}I-labelled ET (7, 9). In the rat adrenal gland the medulla was intensely labelled, but a less dense displaceable binding was also seen in the cortex (9). ET-receptor subtypes (ET_A, ET_B) and ET isopeptides (ET-1 and ET-3) are concomitantly expressed by human adrenal cortex (6). However, as far as we are aware, there has been no report on the topographical distribution of ET-1

in the adrenal gland. The aim of the present investigation was therefore to localize the ET-1 immunoreactivity in the human adrenal gland representing normal tissue or glandular tissue adjacent to different types of adrenal endocrine tumours and in different types of neoplastic adrenal lesions.

Materials and methods

The study material included tissue samples from adrenal glands from 4 patients (1 female (F), 3 males (M); mean age 71 years, range 68–78 years) operated on for neoplasms not engaging the adrenals, and from 33 subjects (25 F, 8 M; mean age 51 years range 23–79 years) from adrenal tissue adjacent to cortical or medullary neoplasms. The former tissue samples are designated *nor-*

Table 1 ET-1 immunoreactivity of adrenal neoplasms (*S*, <2 cm; *M*, 2–5 cm; *L*, >5 cm; *COA*, cortical adenoma; *Phaeo*, phaeochromocytoma; *CC*, cortical carcinoma; *1*, very few; *2*, less than 10%; *3*, 10–50%; *4*, more than 50% of the cells; *, weak staining; **, moderate staining; –, no stained cells)

Patient no.	Sex	Age (years)	Tumour type	Tumour size	Clinical syndromes/ symptoms	Immunoreactivity
2	F	56	COA	M	Cushing's	4/**
4	F	55	COA	M	Cushing's	4/**
5	F	43	COA	M	Cushing's	4/**
6	F	35	COA	M	Cushing's	4/*
13	M	55	COA	M	Cushing's	1/*
16	F	38	COA	M	Cushing's	4/**
7	F	39	COA	S	Conn's	4/**
9	F	50	COA	S	Conn's	4/**
10	F	41	COA	S	Conn's	4/*
11	M	43	COA	S	Conn's	3/*
15	M	40	COA	S	Conn's	2/*
17	M	46	COA	S	Conn's	3/**
19	F	79	COA	S	Conn's	2/**
8	M	50	COA	M	Conn's	4/**
20	F	59	COA	S	Non-functioning	3/**
21	F	68	COA	S	Non-functioning	4/**
1	F	61	COA	M	Non-functioning	4/**
12	M	63	COA	M	Non-functioning	2/**
18	F	60	COA	M	Non-functioning	4/**
23	F	58	COA	M	Non-functioning	4/*
24	F	67	COA	M	Non-functioning	2/*
14	F	64	COA	L	Non-functioning	3/**
31	M	23	COA	L	Non-functioning	3/*
55	F	29	CC	L	Cushing's	–
26	F	62	CC	L	Virilism	–
30	F	59	CC	L	Virilism	–
22	F	65	CC	L	Non-functioning	2/*
25	F	70	CC	L	Non-functioning	–
27	F	67	CC	L	Non-functioning	–
28	F	47	CC	L	Non-functioning	1/*–**
29	F	72	CC	L	Non-functioning	–
56	M	46	CC	L	Non-functioning	1/*
57	M	65	CC	L	Non-functioning	–
32	M	61	Pheo	S	Phaeo-related	–
35	F	55	Pheo	S	Phaeo-related	–
37	M	38	Pheo	S	Phaeo-related	–
39	F	30	Pheo	S	Phaeo-related	–
44	F	57	Pheo	S	Phaeo-related	–
46	F	44	Pheo	S	Phaeo-related	–
33	F	35	Pheo	M	Phaeo-related	–
34	F	73	Pheo	M	Phaeo-related	–
36	F	35	Pheo	M	Phaeo-related	2/*
38	F	77	Pheo	M	Phaeo-related	2/*
42	F	47	Pheo	M	Phaeo-related	–
45	F	57	Pheo	M	Phaeo-related	–
40	M	76	Pheo	L	Phaeo-related	–
41	F	41	Pheo	L	Phaeo-related	–
43	F	51	Pheo	L	Phaeo-related	–
47	F	58	Pheo	L	Phaeo-related	–

mal glandular tissue, and the latter *tumour-free glandular tissue*. The patients contributing normal glandular tissue were operated on for urothelial cancer (M, 68 years), leiomyosarcoma (M, 70 years), renal carcinoma (F, 78 years) and angiolipoma (M, 67 years). Eighteen patients belonging to the latter category had cortical adenoma associated with Cushing's syndrome (4 cases, 3 F, 1 M; mean age 48 years, range 38–56 years), or Conn's syndrome (7 cases, 4 F, 3 M, mean age 49 years, range 39–79 years) or were considered non-functioning (7 cases, 6 F, 1 M, mean age 57 years, range 23–68 years). A further 15 patients had phaeochromocytomas (12 F, 3 M, mean age 51 years, range 30–70 years). Tissue specimens from the following neoplasms were examined: cortical adenomas (23 cases), cortical carcinomas (10 cases) and phaeochromocytomas (16 cases). For details of sex, age at operation and type and size of tumour and clinical syndromes/symptoms, see Table 1.

All tissue specimens were fixed in 10% buffered formalin for 18–24 h at room temperature and processed routinely to paraffin.

Deparaffinized sections, 5 μm thick, were stained with haematoxylin and eosin or immunostained to demonstrate ET-1 or chromogranin A immunoreactivity. This staining was used to confirm the diagnosis of phaeochromocytoma. The avidin-biotin complex (ABC) method was used, with diaminobenzidine as chromogen. The sections were pre-treated with 0.3% hydrogen peroxide in methanol for 30 min to suppress the endogenous peroxide. The primary antiserum was applied to the sections overnight at 4° C. The antiserum used was polyclonal rabbit-anti-human ET-1 from Cambridge Research Biochemicals (CA-08-351, Northwich, Cheshire, England), diluted 1:4,000. The slides were then incubated with a biotinylated goat anti-rabbit IgG (1:100) and with ABC (Vectastain Elite Kit, Vector Laboratories, Burlingame, Calif, USA, 1:100) for 30 min and 60 min respectively, at room temperature. Subsequently, the sections were further processed with the glucose oxidase-DAB-nickel method [12] to enhance the staining intensity. As positive control brain tissue with lesions of multiple sclerosis known to display ET-1 immunoreactivity in human neurones [3] was used. Negative controls were obtained by replacing the primary antibody with the diluent alone, or with a non-immune rabbit serum. Absorption testing was carried out by pre-incubating the ET-1 antibodies with synthetic ET-1, ET-2 and ET-3 (0.5, 2 or 10 nmol ET-1 antigen per ml diluted antibody) at 4° C overnight. The test was performed on normal as well as on neoplastic tissue sections. The endothelin antigens were from Cambridge Research Biochemicals (ET-1: PP-05-2280; ET-2: pp-05-2284; ET-3: pp-05-2285). The intensity of the immunostaining was reduced by 0.5 nmol ET-1, and abolished with 2 and 10 nmol respectively; the other two antigens did not affect the staining.

Cryosections from six of the carcinomas were stained for lipid (Oil Red O method).

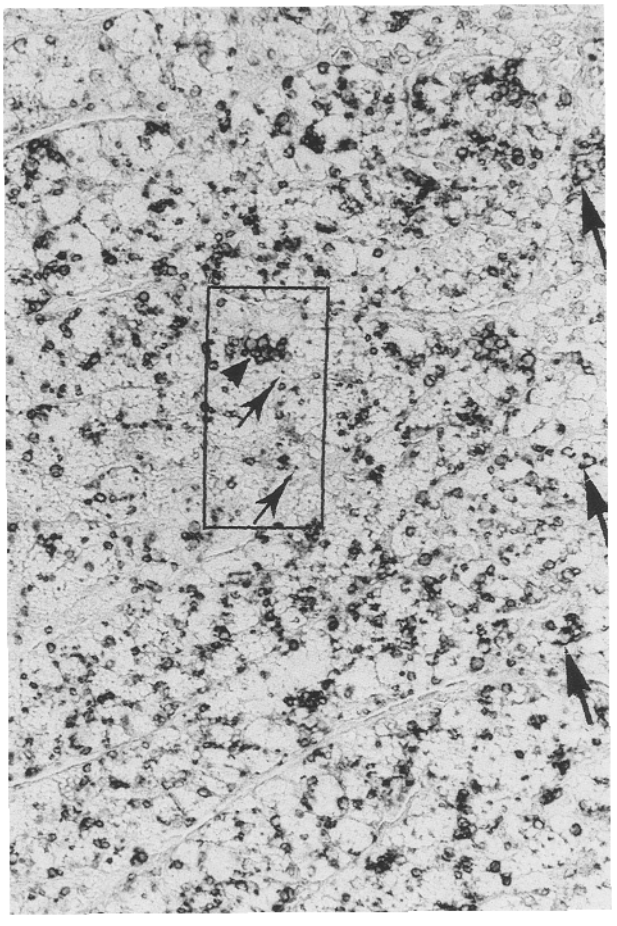

Fig. 1 Photomicrograph showing the ET-1 immunoreactivity in zona fasciculata in the form of vacuoles (*arrowhead*) and grains (*small arrows*) in the cytoplasm. Focally parts of cell membranes (*big arrows*) are stained. Immunostaining vacuoles predominate and occur in most of the parenchymal cells. ABC staining; nickel enhancement. ×236

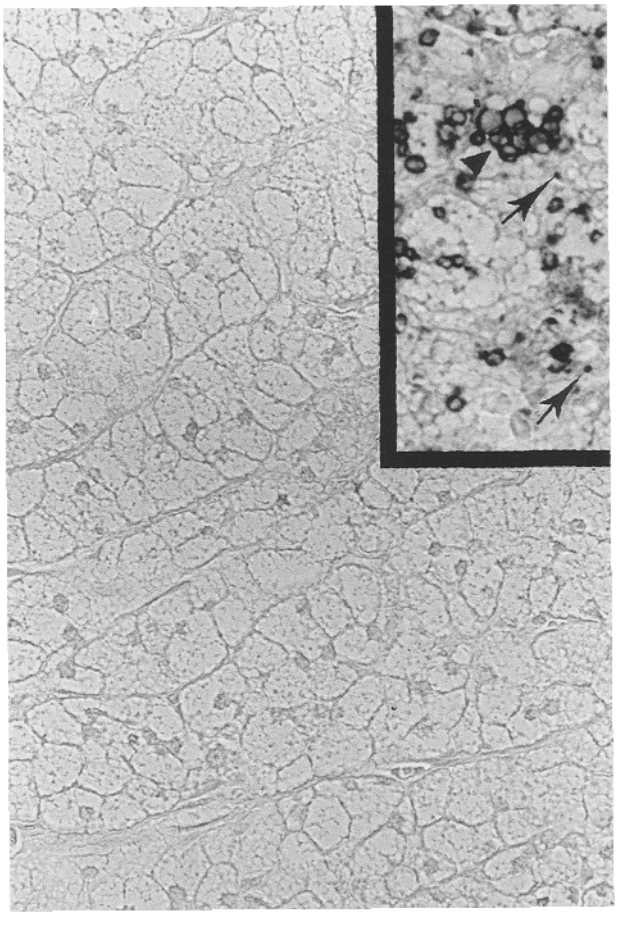

Fig. 2 Photomicrograph from a "negative" control, in which the primary antiserum has been replaced with the diluent alone. ABC staining; nickel enhancement. ×470. Inset: Higher magnification of framed area in Fig. 1. ×470

Results

Normal adrenal tissue

In all cases the cortex contained ET-1 immunoreactive cells, but no reactive cells were seen in the medulla. The reactivity was localized mainly to zona fasciculata, where about half to almost all parenchymal cells displayed immunoreactivity. In the zona glomerulosa the immunoreactive cells were generally few; in the zona reticularis the frequency varied.

The immunoreactivity appeared mainly in the form of stained vacuolar structures in the cytoplasm (Figs. 1, 2). The diameter of these vacuoles varied. Immunoreactive cytoplasmic grains and sporadic immunostained cell membranes were seen in some glandular cells. These grains were found mainly in zona glomerulosa and zona reticularis. No vessels displayed ET-1 immunoreactivity.

Tumour-free adrenal tissue

In the tumour-free adrenal tissue, ET-1 immunoreactivity was localized mainly in the zona fasciculata, where the majority of the cells were stained. In the other two zones there was a wide variation in the frequency of immunostained cells. In the cortex adjacent to 3 of the 7 aldosterone-producing adenomas, immunostained cells were lacking in the zona glomerulosa and in the zona reticularis in one of these cases. There were fewer ET-1-immunoreactive cells in cortical samples from patients with Cushing's syndrome than from individuals with Conn's syndrome, probably because of the occurrence of the cortical atrophy in the former cases. The immunoreactivity appeared in the same forms as seen in the normal subjects. The largest immunoreactive vacuoles were observed in zona fasciculata associated with aldosterone-producing adenoma.

No immunoreactive cells were seen in the medulla.

Cortical adenoma

All 23 adenomas contained ET-1 immunoreactive cells. In 13 of these neoplasms the majority of the parenchymal cells showed immunoreactivity; in 5 cases they accounted for less than 10% of the cell population. The immunoreactive products appeared similar to those occurring in normal and tumour-free cortex but they were generally fewer in number per cell (Fig. 3). There was no

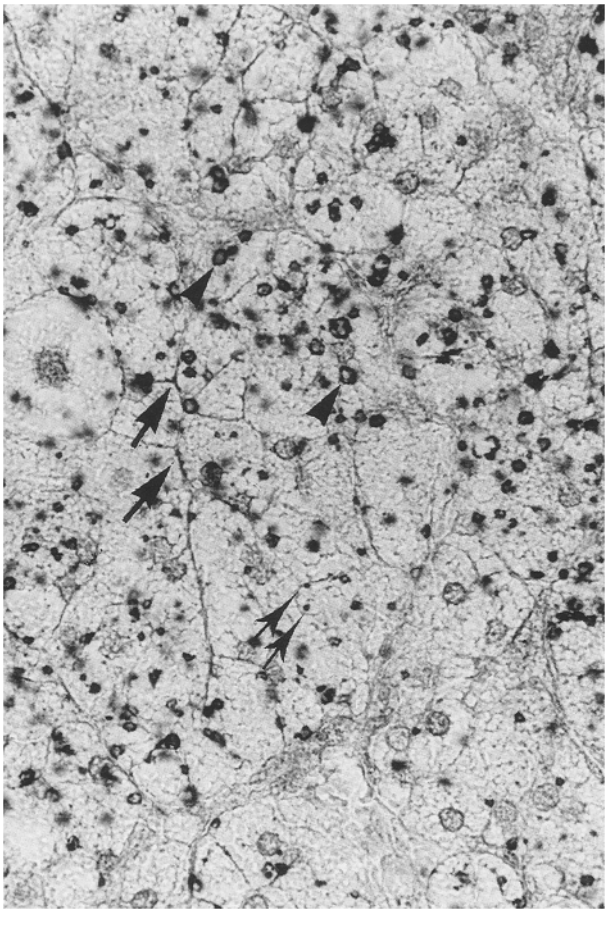

Fig. 3 Photomicrograph showing ET-1 immunostained cells in a cortical adenoma from a patient with Cushing's syndrome. The immunoreactive structures appear as in the normal cortex in the form of vacuoles (*arrowheads*); grains (*small arrows*); cell membranes (*big arrows*), though fewer in number. ABC staining; nickel enhancement. ×470

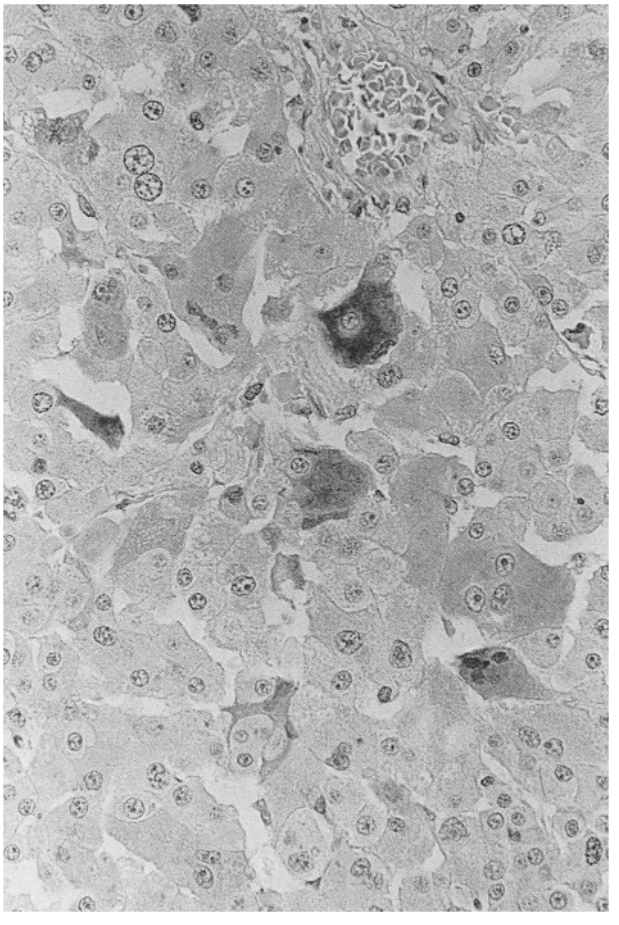

Fig. 4 Cortical carcinoma with a few weakly immunostained parenchymal cells. The immunoreactivity appears as dust-like material. ABC staining; nickel enhancement. ×470

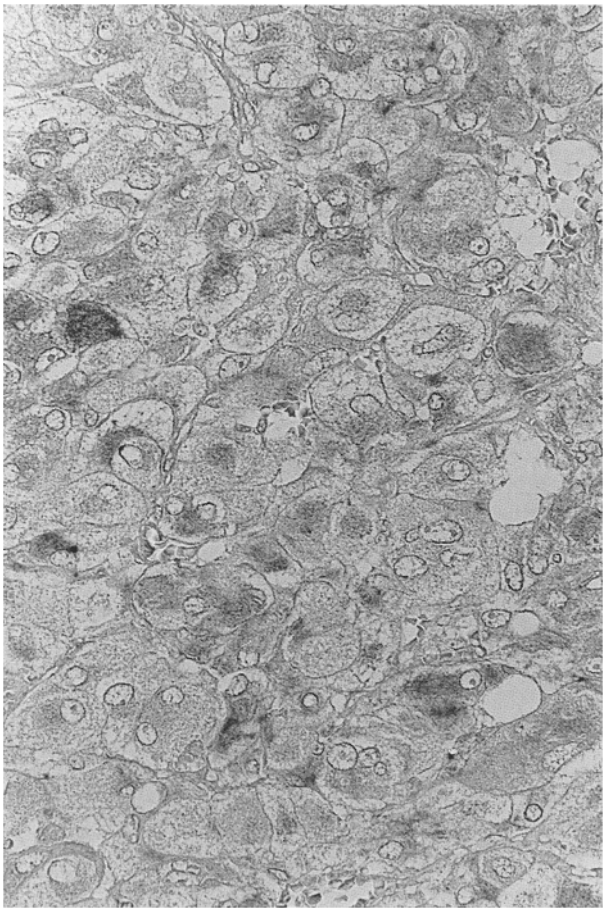

Fig. 5 Phaeochromocytoma displaying an aggregation of neoplastic cells with dust-like immunostained cytoplasm. ABC staining; nickel enhancement. ×470

obvious difference in immunoreactivity between adenomas producing aldosterone and cortisol and those of non-functioning type. Immunostaining was not seen in vessels.

Cortical carcinoma

Three of 10 carcinomas contained sporadic immunoreactive cells, but they were few in number, appeared sporadic and were weakly immunostained. The immunoreactive material appeared mainly as dust-like cytoplasmic material (Fig. 4). In two of the tumours, immunoreactive capillaries were seen; one of them also contained immunoreactive tumour cells.

Fat-containing neoplasmic cells were seen in all carcinomas examined.

Phaeochromocytoma

Two of the 16 phaeochromocytomas contained ET-1 immunoreactive cells but they were few in number, focally localized and only weakly stained (Fig. 5). The immunoreactive material appeared as dust-like structures. ET-1 immunoreactivity was not seen in vessels.

Discussion

Receptor subtypes and isopeptides of ET have earlier been reported to be present in human adrenal cortical adenomas and adjacent normal cortex. These findings have been demonstrated by using a variety of methods: competitive binding technique, and Northern blot analysis of poly(A)$^+$RNA, with cDNAs for ET receptor subtype (ET_A, ET_B) and ET isopeptide (ET-1, ET-3) as probes. These results revealed that ET_A- and ET_B-receptors as well as ET isopeptides were concomitantly expressed in both tissues [6]. However, the analysis techniques used do not permit of any topographical localization of ET-1.

Our immunohistochemical study showed that the antibodies used demonstrated ET-1, but not ET-2 or ET-3, in spite of the similarities in amino acid sequence. The ET-1 reactivity was localized mainly in zona fasciculata, but also to a minor extent in the other two cortical zones. No immunoreactivity could be seen in the medulla. ET-receptor binding sites have, however, been found in the adrenal medulla of rat (9) but, to our knowledge, no report has appeared on the presence of ET-1 in human or rat medulla, using an immunohistochemical technique.

In the present study the immunoreactivity appeared in the cytoplasm in the form of vacuole-like structures, and grains, and was also seen sporadic in the membranes of some cortical parenchymal cells. The nickel treatment enhanced the staining markedly. It has long been known that the cytoplasm of the cells in the zona fasciculata contains membrane-bound vacuoles, ultrastructurally empty-looking, the latter phenomenon being due to the fact that they contain lipids dissolved during the tissue processing (8). Preliminary immuno-electron microscopical studies showed that the immunoreactive product appeared around these vacuoles. Some immunostaining was seen focally on rough endoplasmic reticulum, mitochondrion and focally on the cell membrane (unpublished data). The granular appearance of the immuno products is probably attributable to the endoplasmic reticulum and mitochondrion. Receptor-bound ET-1 has recently been reported (6) in cortical tissue and the focally immunostained cell membranes in our study might be attributable, at least to some extent, to the ET-1 bound by these receptors.

All cortical adenomas in our study contained numerous immunoreactive cells, though there were regional differences. The immunoreactive structures appeared in the same forms as in the normal adrenal cortex but generally less frequently within the cells. No obvious differences in staining pattern could be discerned between the various types of functioning and non-functioning adenomas. However, the immunostaining of the cortical carcinomas differed appreciably from that of the adenomas. Only 3 of the 10 carcinomas contained immunoreactive cells, which were few in number and appeared only fo-

cally. The immunoreactive product occurred only in the form of dust-like material. This immunoreactivity seems not to be related to lipid, as all six carcinomas examined contained fat, but only one tumour revealed dust-like ET-1 immunoreactivity; this difference in immunostaining between the adenomas and carcinomas might be of diagnostic value in distinguishing benign tumours from malignant.

In our study, no ET-1 immunoreactivity could be seen in the adrenal medulla and it appeared in only two of the 16 phaeochromocytomas. The immunoreactive product in these tumours also appeared in a dust-like form and differed from that seen in cortex and cortical adenoma. Our finding is somewhat at variance with that reported by others (13), who found a high concentration of ET-1 in this medullary type of tumour by using radioimmunoassay technique. Whether this difference is due to the sensitivity of the techniques, or the antibodies used, or to a variation in the set of peptides between the tumours is uncertain.

Knowledge of the physiological function of ET-1 in adrenal gland is limited but recent investigations have shown that ET-1 stimulates aldosterone biosynthesis from cultured cells of adrenal zona glomerulosa of calf (1) and secretion from dispersed cells of zona glomerulosa and zona fasciculata in rat and human (4). Furthermore, ET-1 was able to specifically enhance the cell growth and steroidogenic capacity of rat zona glomerulosa (10, 11). ET-1 is further reported to increase aldosterone secretion not only from normal cortex but also from cortical tissue adjacent to the adenoma, but not from the adenoma itself. ET-1 has therefore been proposed as one of the factors that regulate aldosterone secretion in humans (15). The present findings indicating absence of ET-1 immunoreactive cells in zona glomerulosa, in some cases associated with Conn tumours, may further support a relationship between ET-1 and aldosterone, as suggested by Zeng et al. (15). Our finding that ET-1 immunoreactive cells were mainly localized to the zona fasciculata may also support a relationship between this peptide and the glucosteroid regulation. Furthermore, there was no apparent relationship between the frequency and distribution of the ET-1 immunoreactive cells and the different types of tumour tissues. These findings tally with the observation made by Zeng et al. (15) that ET-1 has no effect on the aldosterone secretion in tumour tissue.

In conclusion, our study confirms previously reported findings of ET-1 in human cortex, made by applying biochemical methods (6). We have localized the ET-1 immunoreactivity mainly in parenchymal cells of zona fasciculata, in the form of vacuoles, but also as grains in the cytoplasm and focally on the cell membranes. Irrespective of their hormonal activity, cortical adenomas displayed numerous ET-1-reactive cells, whereas cortical carcinomas generally lacked cells containing ET-1 immunoreactivity. That was also the case for medullary tumours. The difference in ET-1 immunoreactivity between benign and malignant cortical neoplasms may prove to be a valuable tool in histological diagnosis.

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