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# Immunocytochemical demonstration of IGF-II-like immunoreactivity in human paraganglioma of the craniocervical region

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**Summary.** Insulin-like-growth factor (IGF)-II-like immunoreactivity was examined in two carotid bodies and six extra-adrenal paragangliomas with use of monoclonal antibody against rat IGF-II, which crossreacts with human IGF-II. Chief cells but not sustentacular cells of the carotid body were positive at about 10% in one case and less than 1% in another case.

Among four carotid body tumours, a possible vagal body tumour and one glomus jugulare tumour, all but the glomus jugulare tumour exhibited positive tumour cells irrespective of histological variations. The frequency of positive cells ranged from 20 to 60%. IGF-II like immunoreactivity, therefore, might be widely distributed in human extra-adrenal paraganglionic tissues and tumours, although its biological role in these cells remains to be elucidated.

**Key words:** IGF-II – Carotid body – Extra-adrenal paraganglioma – Immunocytochemistry

## Introduction

Paragangliomas are uncommon tumours which originate from extra-adrenal paraganglionic system widely distributed in the body (Glenner 1974). The constituent tumour cells have been postulated to be chief cells in origin. The chief cells are one of the members of APUD cell series (Pearse 1969) or of the paraneuron family, a more recently introduced concept (Fujita 1977). They contain catecholamines (Niemi and Ojara 1964; Kobayashi 1971; Gorgas and Böck 1976; Hansen 1978; Hervonen 1978) and tyrosine hydroxylase, a rate-limiting enzyme in the catecholamine synthesizing pathway (Bolme et al. 1977; Karasawa et al. 1982; Partanen et al. 1984). This enzyme has been found more recently in the paragangliomas of the cervical region (Takahashi et al. 1987). Noradrenalin (Kobayashi et al. 1983) and serotonin (Grönblad et al. 1983) have been also demonstrated in the chief cells and in addition to these amines several peptides including enkephalins (Hansen et al. 1982; Varndell et al. 1982; Kobayashi et al. 1983) and chromagranin A (Kobayashi 1988) have been detected in the cells or their granules. Kobayashi et al. (1983) postulated that a close relationship between carotid body chief cells and adrenal chromaffin cells exists, since met-enkephalin $Arg^6-Gly^7-Leu^8$, a peptide specific to prepro-enkephalin A is present in both cells.

Insulin-like growth factor (IGF) is subdivided into IGF-I and II; IGF-I is a polypeptide composed of 70 amino acid residues and II 67 amino acid residues, respectively (Rindeknecht and Humble 1978). Twenty two amino acid residues among the N-terminal 31 amino acid residues of these two polypeptides, moreover, are identical, and structural homology of the IGFs with insulin and proinsulin has also been reported (Rindeknecht and Humbel 1976, 1978). In Man, increased expression of IGF-II mRNA or increased amount of IGF-II content was detected in fetal kidney, liver, adrenals and striated muscle (Scott et al. 1985). In addition, Wilms tumour (nephroblastoma) of the kidney (Reeve et al. 1985) and pheochromocytoma of the adrenal gland (Haselbacher et al. 1987) have been reported to contain highly expressed IGF-II mRNA or IGF-II. In spite of cellular similarity

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between carotid body chief cells and adrenal chromaffin cells and increased expression of IGF-II mRNA in the adrenal gland, IGF-II content and/or its mRNA has not been studied in paraganglia or paragangliomas to our knowledge. Recently we made an immunocytochemical investigation of IGF-II-like immunoreactivity in two carotid bodies and six paragangliomas of craniocervical region in the human adult. In this paper, we describe the results obtained and discuss their significance.

## Materials and methods

Two normal carotid bodies from 62- and 56-year-old males were obtained at autopsy. The paragangliomas used were summarized in Table 1. All of these tissues were fixed with buffered 10% formalin and embedded in paraffin wax.

Monoclonal antibody of $IG_1$ subtype against rat IGF-II was produced as reported previously (Tanaka et al. 1989). The rat IGF-II produced by a rat epithelial cell line was purified through HPLC and used as an antigen. The cross-reactivity of the antibody with various IGF-related peptides was determined by tracer-binding inhibition in radioimmunoassay using rat IGF-II labeled with $^{125}I$ as a tracer. The results were as follows; human IGF-II; 100%, MSA-III-2 (multiplication stimulating activity-III-2, identical to rat IGF-II, produced by a rat heptocyte cell line BRL-3A (Moses et al. 1987); 100%, human IGF-I; 10%, hEGF; less than 0.01%, and porcine insulin; less than 0.01%.

For immunocytochemistry with the monoclonal antibody from mouse ascites fluid, biotin streptavidin (BSA) method was applied using Biotin-Stereptavidin kit of Bio-Genex Laboratories (Dublin, California, USA). To abolish 10% cross-immuno-

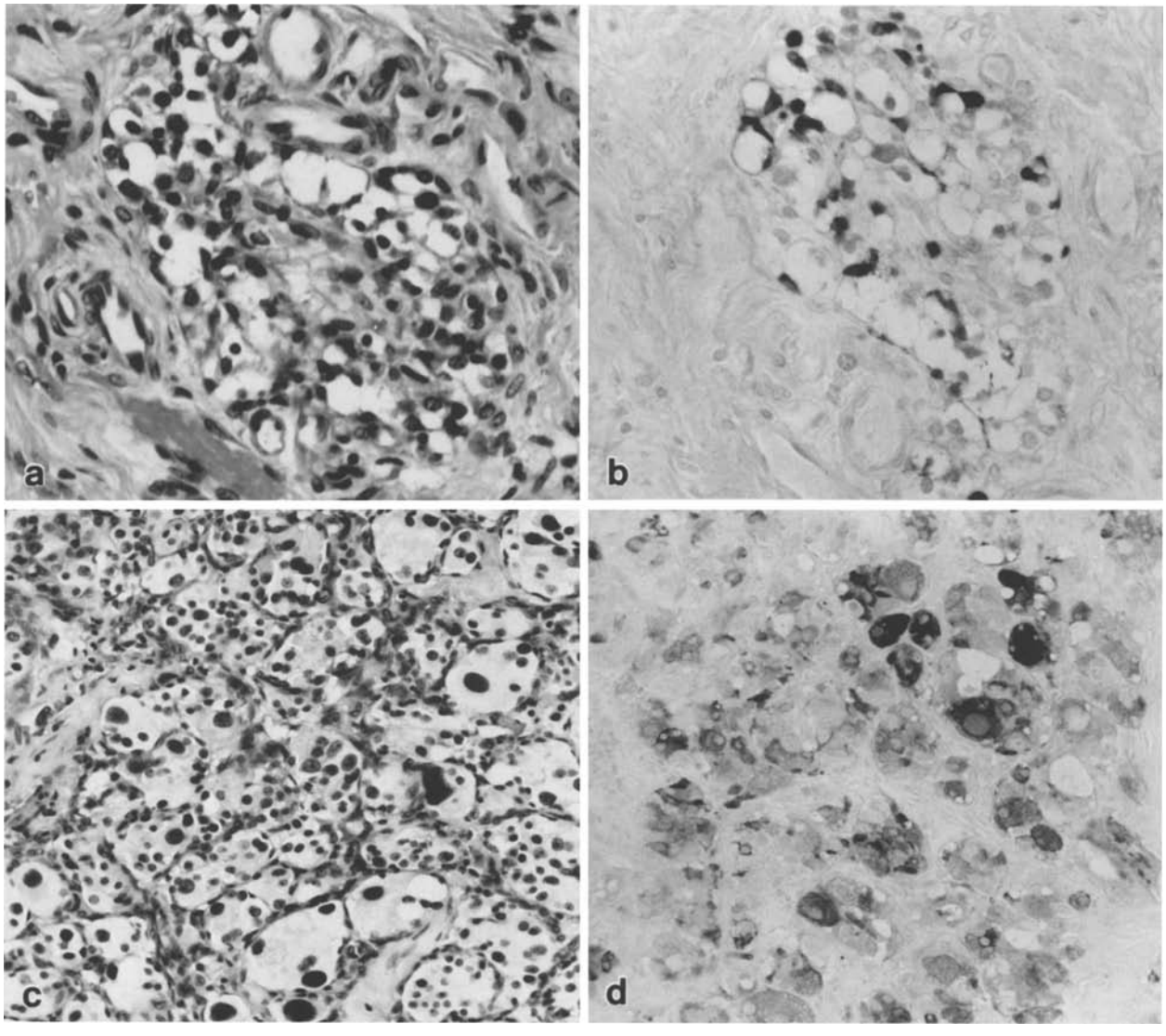


**Fig. 1.** Histology of the normal carotid body reveals chief cells and surrounding sustentacular cells (**a**) (HE, ×200). IGF-II-like immunoreactive cells are found only in chief cell population (**b**) (BSA, ×200). Tumor cells with various sizes (**c**) (HE, ×100, case No. 2) are positively immunostained (**d**) (BSA, ×100, same case)

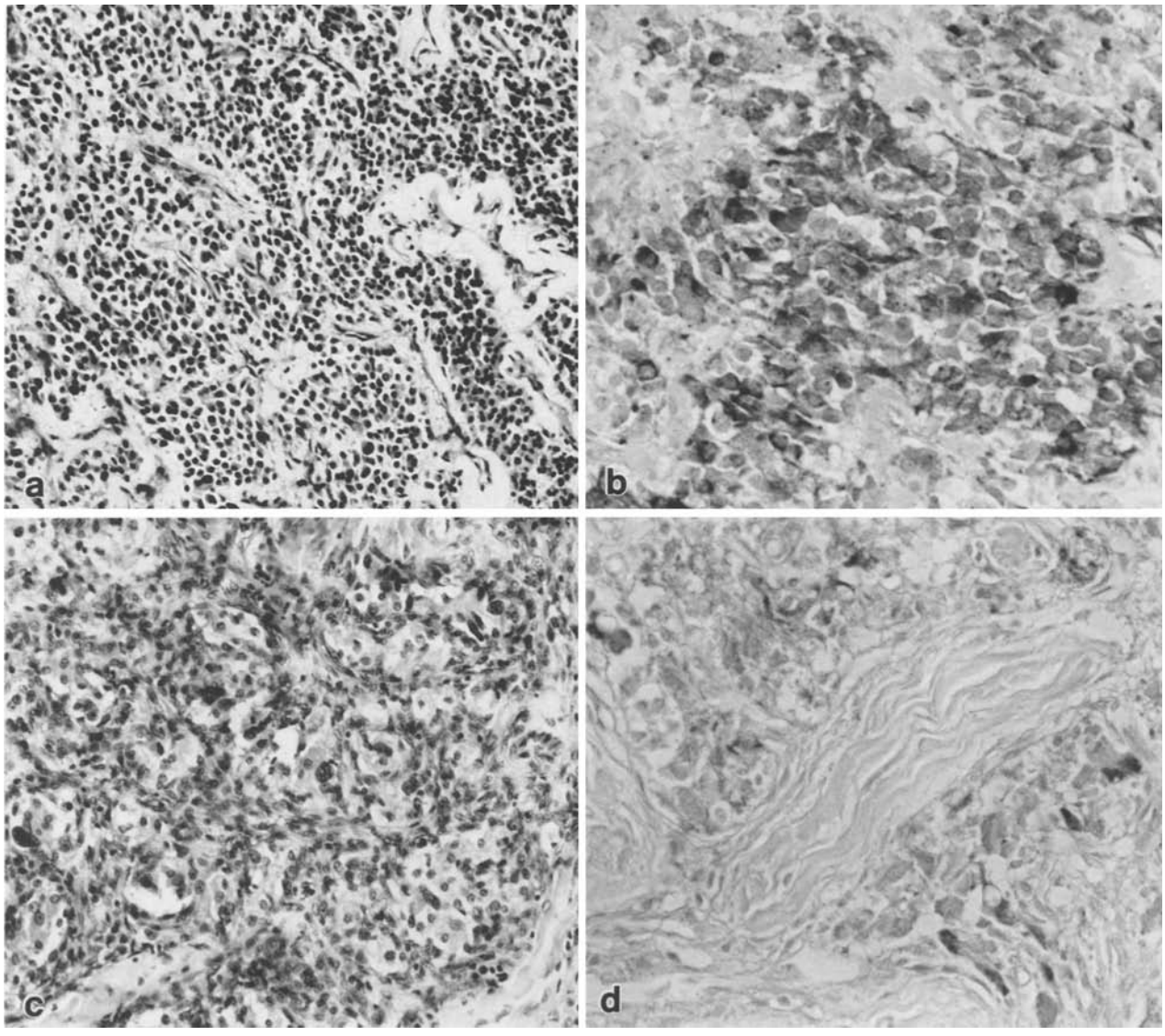


**Fig. 2.** A carotid body tumor composed of small round cells in loose alveolar arrangement (**a**) (HE, ×100) also exhibits IGF-II-like immunoreactive cells with moderate staining intensity (**b**) (BSA, ×200, case No 3. The fourth carotid body tumor is composed of polygonal cells with broad cytoplasm surrounded by fibroblastic cells (**c**) (HE, ×100) and a population of polygonal, not fibroblastic, tumor cells is positively immunostained with weak to moderate staining intensity (**d**) (BSA, ×100)

**Table 1.** List of cases examined

| No. | Age/Sex | Location | Origin | Metastasis |
|---|---|---|---|---|
| 1 | 43/f | lt. neck | carotid body | none |
| 2 | 59/m | rt. sub-mandibular | carotid body | none |
| 3 | 22/m | rt. neck | carotid body | regional node |
| 4 | 28/f | rt. neck | carotid body | unknown |
| 5 | 42/f | lt. neck | vagal body? | none |
| 6 | 60/f | foramen jugulare | rt. glumus jugulare | regional node |

reactivity with IGF-I stated above and possible cross-reaction with proinsulin or c-peptide, the monoclonal antibody was pre-absorbed with one μg of either IGF-I (KabiGen, Stockholm, Sweden), c-peptide (Peninsula Lab., California, USA) or human proinsulin (supplied by N.Y.) before use. Endogenous peroxidase activity was blocked by methanol-hydrogen peroxide for 20 min. After staining with the monoclonal antibody at protein concentration of 0.1 μg/ml over night at 4° C, the slides were treated with biotin-labelled antimouse immunoglobulines, and then streptavidin-conjugated peroxidase for 30 min at room temperature, respectively. Peroxidase reaction product was visualized by Graham-Karnovsky solution (Graham and Karnovsky 1966). Cellular nuclei were stained with 1% methyl green.

The frequency of positively immunostained cells was deter-

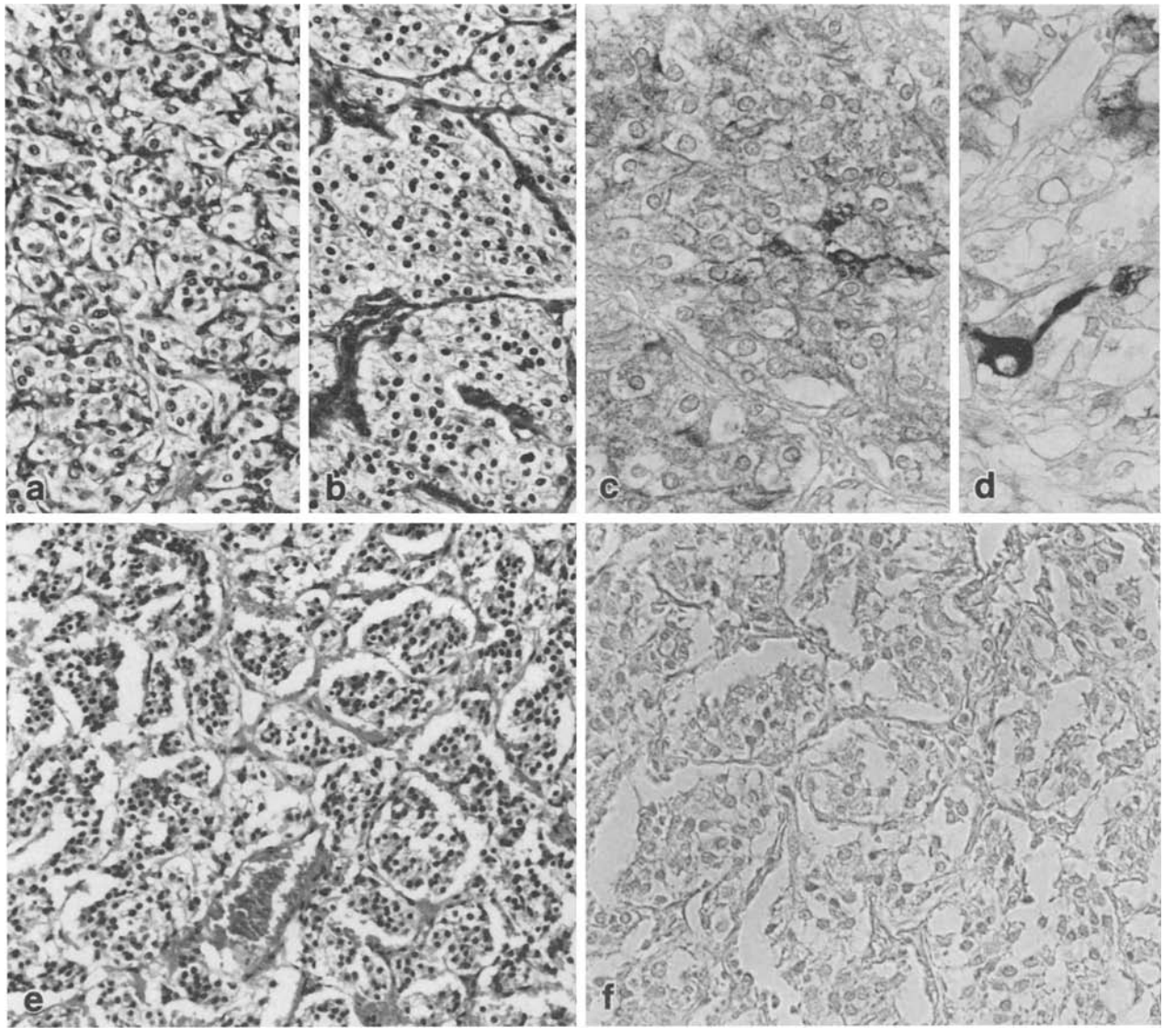


**Fig. 3.** A tumor of possible vagal body origin discloses two histological features; round cells in trabecular arrangement separated by capillary stromy (**a**) and clear round cells in thick trabecular pattern (**b**) (HE, ×100). Mainly weakly, rarely intensely immunostained cells including cytoplasmic processes are found in the former histological area (**c**, **d**) (BSA, ×200). Histology of the jugular body paraganglioma shows small uniform, round tumor cell nests separated by capillary stroma (**e**) (HE, ×100). No immunoreactive cells are detected (**f**) (BSA, ×100)

mined in the area where there were must positive cells. Controls were as follows; omission of the primary antibody and application of the preabsorbed primary antibody with one μg of rat IGF-II in the first step of the immunostaining.

## Results

The normal human carotid body is composed of chief cells, sustentacular cells and neuronal elements with a plentiful fibrovascular stroma (Böck 1982) (Fig. 1a). A part of the chief cells were found to be positive for IGF-II immunoreactivity (Fig. 1b); the frequency of the immunostained cells varied from less than 1% to about 10%.

Four paragangliomas from the carotid body contained positive cells irrespective of their histological variations. Two tumours, cases No. 1 and 2 in Table 1, were composed of polygonal cells with ample cytoplasm and were most frequently populated with IGF-II-like immunoreactive cells, as shown in Figs. 1c and d. The third carotid body tumour was chiefly composed of round and partly polygonal tumour cells arranged in solid acinar pattern (Fig. 2a). The positive cells showed moder-

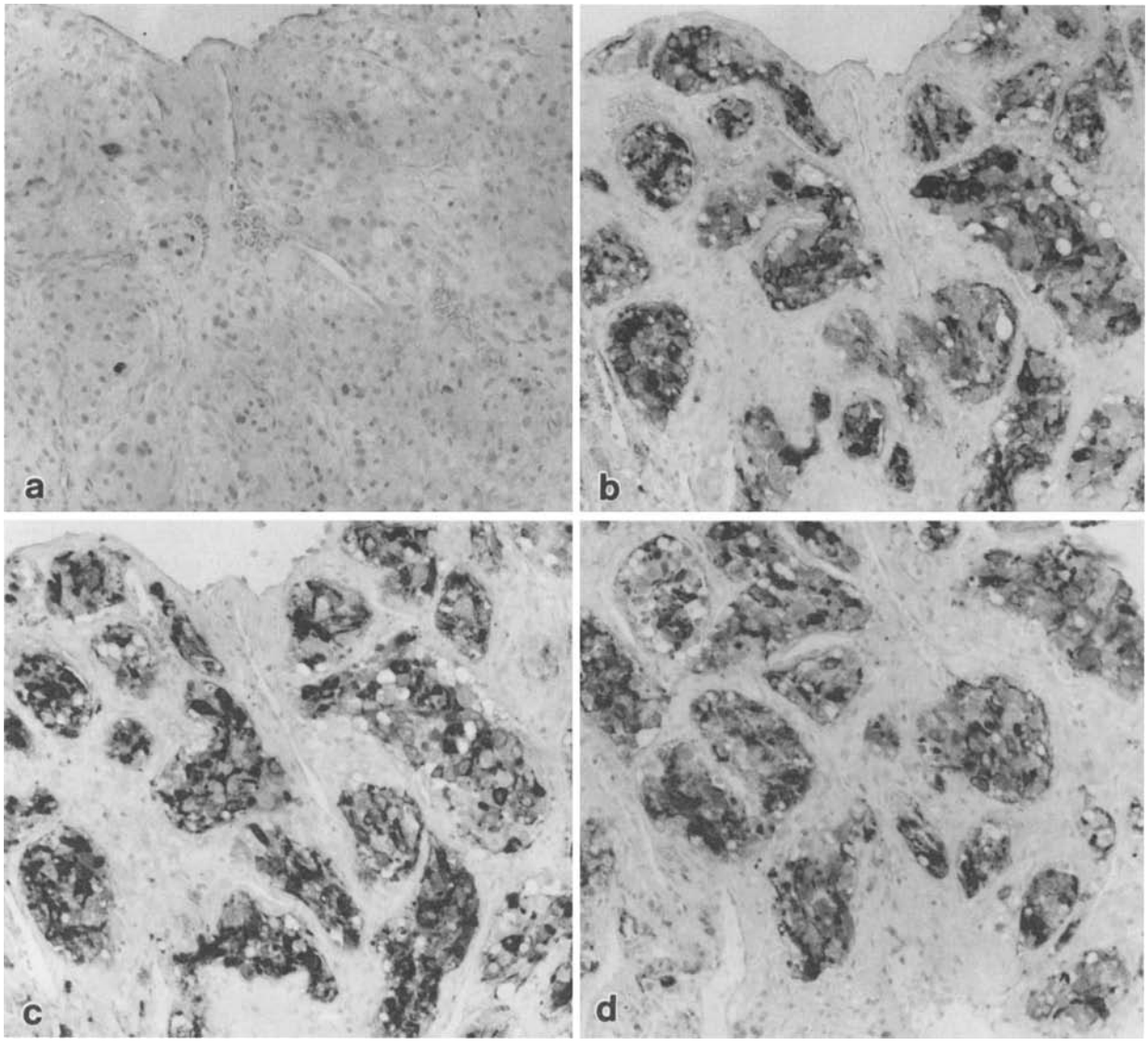


**Fig. 4.** By the immunostaining of carotid body tumor (case No. 2) with the preabsorbed antibody with IGF-II, IGF-I, c-peptide or proinsulin, the positive cells become absent (**a**; IGF-II), or remain present (**b**; IGF-I, **c**; c-peptide; **d**; proinsulin), respectively (BSA, ×100)

ate staining intensity and a focal distribution (Fig. 2b). The fourth carotid body tumour was composed of medium-sized polygonal cells and fibroblastic interstitial cells and arranged in alveolar pattern (Fig. 2c). The positive cells with weak to moderate staining intensity were confined to the polygonal tumour cells, and focally distributed (Fig. 2d).

The tumour attached to the vagal nerve was composed of relatively small, round, uniform cells with oval nuclei. Although the cell borders were indistinct, the cytoplasm was ample and palely eosinophilic, and arranged in trabecular or alveolar pattern with thin vascular septum (Figs. 3a and b). The immunostained cells distributed diffusely and staining intensity was weak (Fig. 3c) but a few of them showed intense immunoreactivity including both cytoplasm and cytoplasmic process (Fig. 3d).

In a tumour found in the lumen of the jugular vein and was considered to be of glomus jugulare origin, the tumour cells were small and uniformly round, and formed regular alveolar pattern with high vascularity. Interstitial cells were not obvious (Fig. 3e). The immunostaining of this specimen, however, failed to disclose positive cells (Fig. 3f).

These results and frequency of positively immunostained cells were summarized in Table 2.

**Table 2.** Distribution and frequency of IGF-II-like immunoreactive cells

| No. | Positive cells | | |
|---|---|---|---|
| | Distribution | Staining intensity | % |
| 1 | diffuse | variable | 61.7 |
| 2 | diffuse | variable | 58.1 |
| 3 | focal | weak to moderate | 34.2 |
| 4 | focal | weak | 28.2 |
| 5 | focal | weak to moderate | 48.5 |
| 6 | none | none | 0 |

The control staining by the preabsorbed antibody with IGF-II gave the negative result (Fig. 4a), and in contrast positive results were obtained by the antibody preabsorbed with either IGF-I, c-peptide or proinsulin as shown in Figs. 4b, c and d.

## Discussion

In the present study, we showed that the chief cells or the carotid body contain IGF-II-like immunoreactivity. In human adrenal glands, the level of IGF-II-mRNA and its content have been reported to be high, and cellular localization of immunoreactivity in the adrenal medulla was also observed by us (unpublished data). Therefore, a close relationship between these two cells has been substantiated. In this context, tumours derived from chief cells of the carotid body might be expected to possess IGF-II-like immunoreactivity. In fact, four paragangliomas of the carotid body, as presented in this study, contained IGF-II immunoreactive cells at about 24 to 60% in frequency irrespective of their histological variations. Moreover, a paraganglioma possibly originating in the vagal body also had IGF-II-like immunoreactive cells at about 50% of tumour cells in frequency. However, one glomus jugulare tumour studied harbored no immunoreactive cells, in spite of its histological resemblance to the carotid body or vagal body paraganglioma. This exceptional result is difficult to explain, but does not exclude the possibility of failure to detect the content of IGF-II-like immunoreactivity in these tumour cells by the present method.

IGF-II has been at first reported to have mitogenic activity in fibroblastic cells (Rechler et al. 1978; Zapf et al. 1987). However, a growth promoting activity via on autocrine mechanism in paraganglioma is not likely, since IGF-II stimulation has not enhanced in vitro cell growth of PC 12, a rat pheochromocytoma cell line (Greene and Tischler 1976) (unpublished observation). Rather, differentiation inducing potency of IGF-II has been reported in chick or rat myoblasts (Schmidt et al. 1983; Ewton et al. 1984) and a cell line of human neuroblastoma (Recio-Pinto and Ishii 1984). In fact the PC 12 differentiates morphologically in vitro, responding to IGF-II stimulation (unpublished data). From these reports and observations, IGF-II seems to induce and maintain differentiated state of the paraganglionic cells. This substance, therefore, might be a "nerve growth factor" of the paraganglionic system, because the nerve growth factor (Levi-Montalcini 1982) has trophic and/or differentiation inducing effect on some sympathetic ganglionic cells. This possibility, of course, remains to be confirmed, but on this point it is interesting that insulin, NGF, relaxin and IGF-II have structural relationships, as pointed out by Bradshaw (1978).

In summary, we report here that paragangliomas of craniocervical region and chief cells of the carotid body harbor IGF-II-like immunoreactivity with high frequency. These results indicate that the IGF-II-like immunoreactivity or IGF-II itself might be widely distributed in both the adrenal and extra-adrenal paraganglionic system.

## References

Böck P (1982) The paraganglia (Handb mikrosk Anat Mensch VI/8). Springer, Berlin, pp 99–104

Bolme P, Fuxe K, Hokfelt T, Goldstein M (1977) Studies on the role of dopamine in cardiovascular and respiratory control: central versus peripheral mechanisms. Adv Biochem Psychopharmacol 16:281–290

Bradshaw RA (1978) Nerve growth factor. Ann Rev Biochem 47:191–216

Dulak NC, Temin HW (1973) A partially purified polypeptide fraction from rat liver cell conditioned medium with multiplication-stimulating activity for embryofibroblast. J Cell Physiol 81:153–160

Ewton DZ, Erwin GB, Pegg AE, Florini JR (1984) The role of polyamines in somatomedin-stimulated differentiation of L6 myoblasts. J Cell Physiol 120:263–270

Fujita T (1977) Concept of paraneurons. Arch Histol Jpn (Suppl) 40:1–12

Glenner GG, Grimley PM (1974) Tumors of the extra-adrenal paraganglion system (including chemoreceptors). Atlas of Tumor Pathology, Series 2, Fascicle 9, Armed Forces Institute of Pathology, Washington, DC

Gorgas K, Böck P (1976) Formaldehyde-induced catecholamine fluorescence in the mouse inferior laryngeal paraganglion. Cell Tissue Res 173:139–142

Graham RC, Karnovsky MJ (1966) The early stage of absorption of injected horseradish peroxidase in proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. A Histochem Cytochem 14:291–302

Greene LA, Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA 73:2424–2428

Gronblad M, Liesi P, Rechardt L (1983) Serotonin-like immunoreactivity in rat carotid body. Brain Res 276:348–350

Hansen JT (1978) Development of type I cells of the rabbit sub-clavian glomera (aortic bodies): a light fluorescence and electronmicroscopic study. Am J Anat 153:15–31

Hansen JT, Brokaw J, Christie D, Karasek M (1982) Localization of enkephalin-like immunoreactivity in the cat carotid and aortic body chemoreceptors. Anat Res 203:405–410

Haselbacher G, Schwab ME, Pasi A, Humbel RE (1985) Insulin-like growth factor II (IGF-II) in human brain: Regional distribution of higher molecular mass forms. Proc Natl Acad Sci USA 82:2153–2157

Haselbacher GK, Irminger J-C, Zapf J, Ziegler WH, Humble RE (1987) Insulin-like factor II in human adrenal pheochromocytomas and Wilms tumors; Expression at the mRNA and protein level. Proc Natl Acac Sci USA 84:1104–1106

Hervonen A, Partanen S, Vaalasti A, Partanen M, Kanerva L, Alho H (1987) The distribution and endocrine nature of the abdominal paraganglia of the adult man. Am J Anat 153:563–572

Karasawa N, Kondo Y, Nagatsu I (1982) Immunohistochemical and immunofluorescent localization of catecholamin-synthesizing enzymes in the carotid body of the rat and dog. Arch Histol Jpn 45:429–435

Kobayashi S (1971) Comparative cytological studies of the carotid body. 1. Demonstration of monoamine storing cells by correlated chromaffin reaction and fluorescence histochemistry. Arch Histol Jpn 33:319–339

Kobayashi S, Uchida T, Ohashi T, Fujita T, Nakao K, Yoshimasa T, Imura H, Mochizuki T, Yanaihara C, Yanaihara N, Verhorstad AAJ (1983) Immunocytochemical demonstration of the costorage of noradrenalin with Met-enkephalin Arg$^6$-Phe$^7$ and Met-enkephalin-Arg$^6$-Gly$^7$-Leu$^8$ in the carotid body chief cells of the dog. Arch Histol Jpn 46:713–722

Kobayashi S (1988) Carotid body chief cells. In: Fujita T, Kanno T, Kobayashi S (eds) The paraneuron. Springer, Berlin Heidelberg New York Tokyo, pp 198–206

Levi-Montalcini R (1982) Developmental neurobiology and the natural history of nerve growth factor. Ann Rev Nerosci 5:341–362

Levitt M, Spector S, Sjoerdsma A, Udenfriend S (1965) Elucidation of the rate-limiting step in norepinephrin biosynthesis in the perfused guinea pig heart. J Pharmacol Exp Ther 148:1–8

Moses AC, Nissley SP, Short PA, Rechler MM, Podskalny JM (1980) Purification and characterization of multiplication-stimulating activity. Eur J Biochem 103:387–400

Niemi M, Ojala K (1964) Cytochemical demonstration of catecholamines in the human carotid body. Nature 203:539–540

Partanen M, Rapoport SI, Reis DJ, Joh TH, Stolk JM, Linnoila I, Teitelman G, Harvonen A (1984) Catecholamine-synthesizing enzymes in paraganglia of aged Fischer-344 rats. Immunohistochemistry and fluorescence microscopy. Cell Tissue Res 238:217–220

Pearse AGE (1969) The cytochemistry and ultrastructure of polypeptide hormone-producing cells of the APUD series and the embryologic, physiologic and pathologic implications of the concept. J Histochem Cytochem 17:303–313

Rechler MM, Flyklund L, Nissley SP, Hall K, Podskalny JM, Skotter A, Moses AC (1978) Purified human somatomedin A and rat multiplication stimulating activity. Mitogens for cultured fibroblasts that crossreact with the same growth peptide receptors. Eur J Biochem 82:5–12

Recio-Pinto E, Ishii DN (1984) Effect of insulin, insulin-like growth factor-II and nerve growth factor on neurite outgrowth in cultured human neuroblastoma cells. Brain Res 302:323–334

Reeve AE, Eccles MR, Wilking RJ, Bell GL, Millow LJ (1985) Expression of insulin-like growth factor-II transcripts in Wilms' tumor. Nature 317:258–262

Rindeknecht E, Humbel RE (1978) Primary structure of human insulin-like growth factor II. FEBS Lett 89:283–286

Rindeknecht E, Humbel RE (1976) Aminoterminal sequence of two polypeptides from human serum with nonsuppressible insulin-like growth-promoting activities: Evidence for structural homology with insulin B chain. Proc Natl Acad Sci USA 73:4379–4381

Rindeknecht E, Humble RE (1978) The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. J Biol Chem 253:2769–2776

Schmidt CH, Steiner TH, Froesch ER (1983) Preferential enhancement of myoblast differentiation by insulin-like growth factors (IGF-I and IGF-II) in primary cultures of chicken embryonic cells. FEBS Lett 161:117–129

Scott J, Cowell J, Robertson ME, Priestley LM, Wadey R, Hopkins B, Pritchard J, Bell GI, Rall LB, Graham DG, Knott TJ (1985) Insulin-like growth factor-II gene expression in Wilms' tumor and embryonic tissues. Nature 317:260–262

Takahashi H, Nakashima S, Kumanishi T, Ikuta F (1987) Paragangliomas of the craniocervical region. An immunohistochemical study on tyrosine hydroxylase. Acta Neuropathol (Berl) 73:227–232

Tanaka H, Asami O, Hayano T, Sasaki I, Yoshitake Y, Nishikawa K (1989) Identification of a family of insulin-like growth factor II secreted by cultured rat epithelial cell line, 18, 54-SF: Application of a monoclonal antibody. Endocrinology 124:870–877

Varndell IM, Tapia FJ, De Mey J, Rush RA, Bloom SR, Polak JM (1982) Electron immunocytochemical localization of enkephalin-like material in catecholamine-containing cells of the carotid body, the adrenal medulla, and in pheochromocytomas of man and other mammals. J Histochem Cytochem 30:682–690

Zapf J, Schoenle E, Froesch R (1978) Insulin-like growth factors I and II: Some biological actions and receptor binding characteristics of two purified constituents of nonsuppresible insulin-like activity of human serum. Eur J Biochem 87:285–296

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