Identification of the Secretogranin II-Derived Peptide EM66 in Pheochromocytomas as a Potential Marker for Discriminating Benign Versus Malignant Tumors

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CHROMOGRANINS (Cgs) CONSTITUTE a family of acidic glycoproteins whose major members are chromogranin A (CgA), chromogranin B (CgB), and secretogranin II (SgII). All Cgs are stored within large dense-core vesicles of neurons and endocrine cells (1–3), making these proteins useful markers for the identification of neuroendocrine cells and neoplasms (4, 5). Cgs are characterized by a high proportion of acidic amino acids and by the presence of multiple pairs of basic residues which represent potential cleavage sites by prohormone convertases. This latter characteristic strongly suggests that Cgs may serve as precursors to generate biologically active peptides. In particular, secretoneurin (SN), a 33-amino acid peptide, has been identified as an authentic processing product of SgII in different species (6, 7). It has been subsequently found that SN occurs in a variety of tissues and exerts diverse biological activities (for a review, see Ref. 8). Comparison of the cDNA sequences of SgII in human and amphibians (9–11) has shown that only two domains bounded by dibasic residues have been highly conserved during evolution, *i.e.* SN and a 66-amino acid C-terminal flanking peptide termed EM66. Using specific polyclonal antibodies directed against recombinant human EM66, we have previously shown that this peptide is actually generated in chromaffin cells of fetal and adult human adrenal glands (12).

While the functions of Cgs are still not fully understood, CgA has been established as a useful marker molecule for neuroendocrine tumors (for a review, see Ref. 13). Similarly, SgII occurs in various tumors such as neuroblastomas, carcinoids, thyroid carcinomas, pituitary adenomas, and pheochromocytomas (14–17). Tissue CgA and SgII concentrations differ depending on the nature of the neuroendocrine tumors (15, 18), indicating that CgA- and SgII-derived peptides can be used as clinical markers for the diagnosis and prognosis of these neoplasms. Indeed, it has been reported that the CgA-derived peptide vasostatin I may help to distinguish between metastatic deposits originating from ileon or lung carcinoid primary tumors (19). It has also been shown that elevated plasma SN concentrations are associated with several endocrine tumors (20) and progression of prostatic carcinoma (21).

The aim of the present study was to localize and characterize the novel SgII-derived peptide EM66 in a series of human pheochromocytomas to evaluate its clinical usefulness as a biological marker of this neuroendocrine tumor.

Patients and Methods

Patients and tissue collection

The presence of EM66-immunoreactivity was investigated in 10 pheochromocytomas, 6 benign tumors (patients 1–6 in Table 1), and 4 ma-

Abbreviations: Cg, Chromogranin; CgA or CgB, Cg A or B; PB, phosphate buffer; SgII, secretogranin II; SN, secretoneurin; TFA, trifluoroacetic acid; TH, tyrosine hydroxylase.

TABLE 1. Clinical characteristics of patients with pheochromocytoma

	Patient		Pheochromocytoma		
	Sex	Age	Status	Site	Diameter (mm)
1	M	46	Be	Left adrenal	30
2	M	51	Be	Zuckerkandl body	38
3	M	60	Be	Left adrenal	43
4	M	65	Be	Right adrenal	50
5	F	68	Be	Right adrenal	80
6	F	67	Be	Right adrenal	40
7	F	46	Ma	Right adrenal	45
8	F	18	Ma	Zuckerkandl body	50
9	M	43	Ma	Urinary bladder	50
10	F	54	Ma	Right adrenal	140

Patients 7 and 8 had benign tumors at initial surgery and subsequently developed metastatic recurrences. In these two patients, studies were performed on pheochromocytoma metastases. Be, Benign; F, female; M, male; Ma, malignant.

lignant tumors (patients 7–10 in Table 1). The tissues were provided by a French endocrinological network for collection of adrenal tumors (Réseau COMETE-2, PHRC AOM 02068). The patients gave written informed consent and the protocol of collection of the tissue was approved by the regional bioethics committees (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Haute-Normandie et de Paris, France). After collection, tissues were kept frozen at −80 C. Clinical characteristics of patients and tumors are reported in Table 1.

Immunohistochemical procedure

Frozen tissues were sliced into 10-μm sections with a cryomicrotome (Frigocut 2800E, Reichert-Jung, Nussloch, Germany). Sections were thaw-mounted on gelatin-coated slides, dried in a desiccator overnight at 4 C, and dipped for 10 min in Stephanini's fixative (4% paraformaldehyde and 0.2% picric acid in phosphate buffer, PB). After several rinses in PB, slices were processed either for indirect immunofluorescence or immunoenzymatic microscopy as previously described (12). The immunofluorescence technique was applied for colocalization experiments using two different fluorochromes. Briefly, tissue sections were incubated overnight at 4 C with an EM66 antiserum directed against human EM66 (code no. 736-1806) diluted 1:200 in PB containing 0.3% Triton X-100 and 1% BSA. The tissues were rinsed in PB and then incubated for 90 min at room temperature with fluorescein isothyocyanate-conjugated goat antirabbit γ-globulins (Caltag Laboratories, Inc., San Francisco, CA) diluted 1:100. For colocalization studies, tissue sections were incubated simultaneously with the EM66 antiserum and a mouse monoclonal antibody against tyrosine hydroxylase (TH; Bioproducts, Gagny, France) diluted 1:200, and the immunoreactivity was revealed with fluorescein isothyocyanate-conjugated goat antirabbit γ-globulins and Texas Red-conjugated goat antimouse γ-globulins (Amersham Pharmacia Biotech, Buckinghamshire, UK). Finally, tumor slices were rinsed in PB, mounted with buffer/glycerol (1:1), coverslipped, and observed on an Orthoplan microscope (Leitz, Heidelberg, Germany) equipped with a Vario-Orthomat photographic system or on a confocal laser scanning microscope (Leica Corp., Heidelberg, Germany) equipped with a Diaplan optical system and an argon/krypton ion laser (excitation wavelengths: 488/568/647 nm). Immunoenzymatic labeling of pheochromocytoma slices was performed using a commercial kit (EnVision+ System, Peroxidase; DAKO Corp., Carpinteria, CA). The tissue slices were incubated for 5 min with a 0.03% hydrogen peroxide solution to quench any endogenous peroxidase activity. After several rinses in distilled water, the slides were incubated for 30 min with the EM66 antiserum diluted 1:5000 in Tris-buffered saline (0.05 M Tris-HCl; 0.15 M NaCl, pH 7.4; TBS) containing 0.3% Triton X-100 and 1% BSA. Slices were rinsed in TBS and then incubated for another 30 min with a peroxidase-labeled polymer conjugated to goat antirabbit γ-globulins. Finally, a 3, 3′-diaminobenzidine chromogen solution was applied for 2–3 min, the tissues were rinsed in distilled water, counterstained with hematoxylin, and mounted with Eukitt medium.

To verify the specificity of the immunoreaction, the following controls were performed: 1) substitution of the primary antibodies with PB; 2) incubation with nonimmune rabbit serum instead of the EM66 antisera; and 3) preincubation of the EM66 antiserum (diluted 1:200 or 1:5000 for, respectively, immunofluorescent or immunoenzymatic methods) with purified recombinant EM66 (10^{-6} mol/liter).

Tissue extraction

For reversed-phase HPLC analysis and RIA, frozen tissue samples were boiled for 10 min in 0.5 M acetic acid as previously described (18), homogenized in a glass Potter, sonicated and centrifuged (3000 $\times$ *g*, 4 C) for 30 min. The supernatants were collected and kept at 4 C until prepurification. The pellets were used for measurement of protein concentrations by the Lowry method.

For Western blot analysis, normal adrenal glands and pheochromocytoma tissues were homogenized in 10 mM Tris-HCl (pH 7.4), containing 0.05% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. After centrifugation (21,000 $\times$ *g*, 4 C) for 15 min, the proteins contained in the supernatants were analyzed.

SDS-PAGE and Western blot analysis

Proteins were analyzed by PAGE under denaturing conditions (22), electroblotted onto nitrocellulose membranes (Amersham Pharmacia Biotech) and revealed with EM66 antibodies as previously described (12), using a chemiluminescence detection kit (Amersham Pharmacia Biotech).

Prepurification of tissue extracts

The supernatant from each tumoral tissue extract was loaded onto a Sep-Pak C_{18} cartridge (Waters Corp., St-Quentin en Yvelines, France) equilibrated with a solution of 0.1% trifluoroacetic acid (TFA) in water. Bound material was eluted from the cartridge with acetonitrile/water/TFA (59.9:40:0.1, vol/vol/vol), dried by vacuum centrifugation (Speed-Vac Concentrator, Savant AES 2000, Hicksville, NY) and kept at room temperature until chromatographic analysis or RIA.

RIA

EM66 RIA was performed using the same antiserum as that employed for immunohistochemistry (code no. 736-1806). Purified recombinant EM66 was iodinated by the chloramine-T method and separated from free iodine on Sep-Pak C_{18} cartridges using a gradient of acetonitrile (0–100%) in 0.1% TFA, as previously described (12). The assay was performed in veronal buffer (pH 7.4) supplemented with 0.4% BSA and 0.1% Triton X-100. The antiserum, used at a final dilution of 1:30,000, was incubated with 7,000 cpm of tracer/tube for 48 h at 4 C in the presence of graded concentrations of standard (purified EM66), tissue extracts, or HPLC fractions. The antibody-bound fraction was immunoprecipitated by addition of 200 μl goat antirabbit γ-globulins (1:30), 200 μl normal rabbit serum (1:100), and 1 ml of a 20% polyethylene glycol 8000 solution. After a 2-h incubation at room temperature, the mixture was centrifuged and the pellet containing the bound fraction was counted on a gamma counter (LKB-Wallac, Inc., Rockville, MD). The standard curve was set up with concentrations of EM66 ranging from 5–10,000 pg/tube.

HPLC analysis

Dried samples were reconstituted in 0.5 ml acetonitrile/water/TFA (9.9:90:0.1, vol/vol/vol; solution A), centrifuged (21,000 $\times$ *g*) and injected onto a 4.6 $\times$ 250 mm Vydac 218TP54 column equilibrated with solution A at a flow rate of 1 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 60% over 25 min using a linear gradient. One-microgram samples of purified recombinant EM66, used as HPLC reference standard, were chromatographed in the same conditions. Fractions of 0.5 ml were collected, evaporated and kept dry until RIA.

Data analysis

Differences between medians of EM66 concentrations in the two groups of patients with benign or malignant pheochromocytomas were

assessed using the Mann-Whitney test. Data were analyzed with the Prism program (GraphPad Software, Inc., San Diego, CA). The displacement curves were fitted using the Prism program.

Results

Western blot analysis and immunohistochemical detection of EM66

As previously reported (12), the EM66 antiserum revealed the presence, in the normal human adrenal gland, of a SgII-immunoreactive band that migrated at a position corresponding to an apparent molecular mass of 97K, in addition to several bands of lower mass (Fig. 1). In two malignant pheochromocytomas, several similar bands were observed indicating that the EM66 antibodies recognize SgII and its processing products in tumoral chromaffin tissues (Fig. 1). EM66 immunoreactivity was also detected by immunohistochemistry in sections of the ectopic vesical pheochromocytoma (patient 9 in Table 1; Fig. 2A). The EM66 immunoreactivity was observed in cell clusters and confined to the cytoplasm (Fig. 2B). Double-labeling experiments, using the EM66 antiserum (Fig. 2B) and the TH antibodies (Fig. 2C), revealed that the EM66-immunoreactive material was restricted to chromaffin cells. Immunoenzymatic labeling of sections from a benign pheochromocytoma (patient 4 in Table 1; Fig. 2, D and E) produced intense staining of tumoral cells. Preabsorption of the EM66 antiserum with 10^{-6} M recombinant EM66 totally abolished the immunoreaction (Fig. 2F). When the antiserum was substituted either with nonimmune rabbit serum or with PB, no immunostaining was observed.

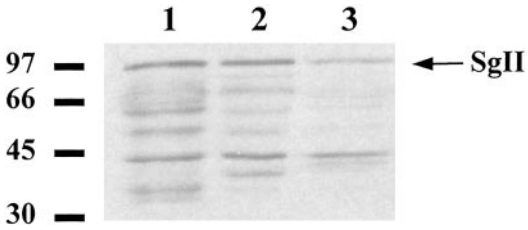

FIG. 1. Western blot analysis of a human adrenal gland (patient 1, lane 1) and malignant pheochromocytoma (patient 8, lane 2; and patient 10, lane 3) extracts. Tissue samples were homogenized, and proteins (30 μg) were analyzed by SDS-PAGE followed by immunoblotting using the EM66 antiserum at a 1:4,000 dilution. Molecular mass markers (in kilodaltons) and the SgII position are indicated.

Characterization of EM66-like material in pheochromocytoma extracts

Serial dilutions of benign pheochromocytoma extracts generated displacement curves that were parallel to that obtained with recombinant EM66 (Fig. 3A). Similar results were obtained with the four malignant tissue extracts (Fig. 3B).

The scattergram of EM66 concentrations for each tumor extract is shown in Fig. 4. In patients with benign tumors,

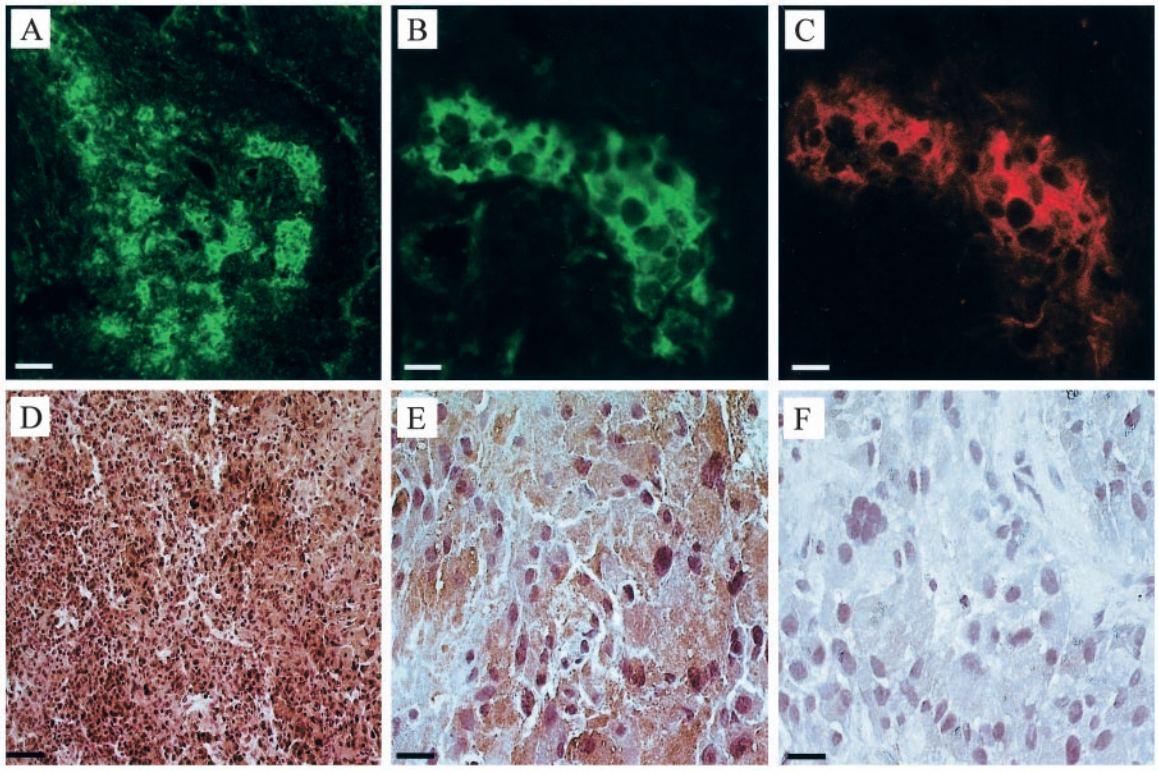

FIG. 2. Immunohistochemical labeling of EM66 in malignant and benign pheochromocytomas. A, Intense staining was observed in clusters of cells dispersed among the vesical tissue (patient 9 in Table 1). B and C, Close-up view of the same tissue section coincubated with the EM66 antiserum (B) and with a monoclonal antibody against TH (C) showing that the immunostaining is confined to chromaffin cells. D and E, Dense EM66-immunoenzymatic labeling of sections of a benign pheochromocytoma (patient 4 in Table 1) counterstained with hematoxylin. F, Preabsorption of the EM66 antiserum with purified recombinant EM66 (10^{-6} mol/liter) totally abolished the immunostaining. *Bars*, 50 μm (A); 12.5 μm (B and C); 60 μm (D); and 15 μm (E and F).

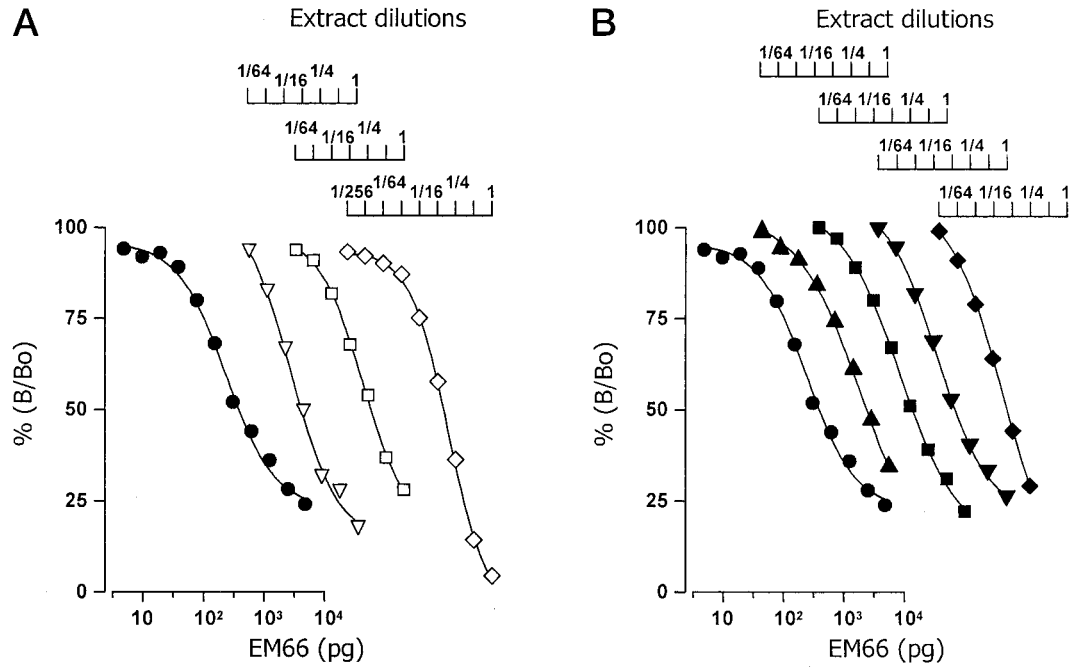

FIG. 3. Semilogarithmic plots comparing competitive inhibition of antibody-bound ^{125}I-EM66 by recombinant EM66 (●) and serial dilutions of pheochromocytoma extracts. Displacement curves were obtained with A, benign (▽, patient 1; □, patient 2; ◇, patient 3); and B, malignant tumor extracts (▲, patient 7; ■, patient 8; ▼, patient 9; ◆, patient 10).

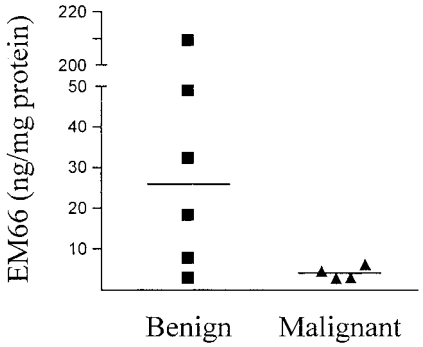

FIG. 4. Scattergram of EM66 concentrations in benign (■) and malignant (▲) pheochromocytoma extracts. The *bars* represent the median value for each group.

EM66 concentrations ranged from 3.2–210 ng/mg protein with a median value of 25.6 ng/mg protein (3.25 pmol/mg protein). The concentrations of EM66 immunoreactivity in malignant tumors ranged from 2.9–6.3 ng/mg protein with a median value of 3.8 ng/mg protein (0.48 pmol/mg protein). Statistical analysis using the nonparametric Mann-Whitney test revealed that the concentration of EM66 in benign tumors was significantly higher than in malignant tumors ($P < 0.05$).

Biochemical characterization of EM66-immunoreactivity in the different pheochromocytomas was performed by HPLC analysis combined with RIA quantification (Fig. 5). Recombinant EM66 eluted in fractions 34–36, 35–37, or 36–38 (44–46%, 45–47%, or 46–48% acetonitrile) depending on the day of the experiment. All benign tumors analyzed contained a major immunoreactive peak coeluting with recombinant EM66 (Fig. 5, A–C). In three malignant pheochromocytoma extracts, a single immunoreactive peak coeluting with the recombinant peptide was also resolved (Fig. 5, D–F).

Discussion

Within the SgII protein, the sequences of the two peptides SN and EM66 have been strongly preserved during evolution (10, 11), suggesting that these novel peptides fulfill important functions. We have previously shown that EM66, like SN (23), is present in the normal human adrenal gland (12). The present study now demonstrates the occurrence of EM66 in human pheochromocytomas.

Double labeling of a vesical pheochromocytoma with EM66 and TH antibodies revealed that EM66 immunoreactivity is confined to chromaffin cells. Analysis of two malignant pheochromocytoma extracts by Western blotting indicated that the EM66 antibodies recognize to some extent human SgII and intermediate processing products. In a series of six benign or malignant pheochromocytoma extracts, HPLC analysis combined with RIA detection resolved a single immunoreactive peak coeluting with recombinant EM66. These data demonstrate that, in adrenomedullary neoplasms as well as in extra-adrenal pheochromocytomas, SgII serves as a precursor to generate the peptide EM66. In agreement with this observation, previous studies have shown that, in pheochromocytomas, ganglioneuromas, and neuroblastomas, SgII is processed to form SN (18, 24, 25). It thus appears that processing of SgII at the Lys-Arg dibasic sites flanking EM66 actually occurs in tumoral endocrine tissues.

The occurrence of Cgs in secretory granules makes these proteins useful markers for the identification of neuroendocrine cells and neoplasms (4, 5). In particular, measurement of plasma CgA levels has long been used for the diagnosis of gut carcinoids and pancreatic islet-cell tumors (for a re-

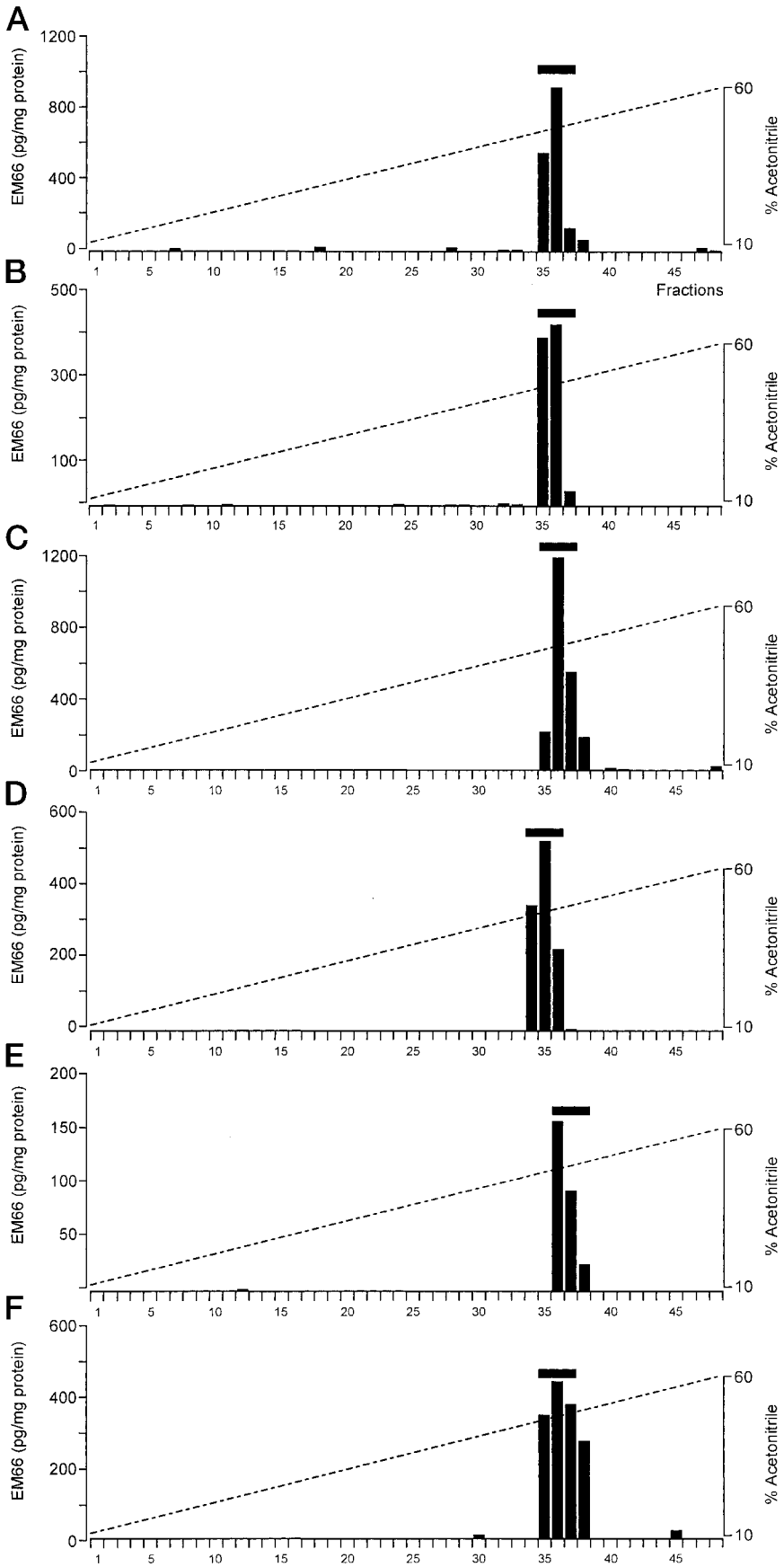

FIG. 5. Reversed-phase HPLC analysis of EM66-immunoreactivity in benign (A–C, patients 1–3, respectively) and malignant pheochromocytomas (D–F, patients 7, 9 and 10, respectively). Tissue extracts were prepurified on Sep-Pak cartridges and chromatographed onto a Vydac C_{18} column. Fractions were collected (0.5 ml/tube), dried, and radioimmunoassayed for EM66. The *bars* above the peaks indicate the elution position of recombinant EM66 chromatographied the same day as the tissue extracts. The *dashed lines* show the concentration of acetonitrile in the eluting solvent.

view see, Ref. 13). However, several studies have shown that neuroendocrine tumors contain either comparable amounts of CgA and SgII (15, 16, 26, 27) or higher concentrations of SgII as for ganglioneuromas and neuroblastomas (18). In addition, Baudin *et al.* (28), recommended that CgA should not be used as a marker for some tumors such as medullary thyroid carcinoma. On the other hand, elevated serum levels of the SgII-derived peptide SN have been shown to be associated with several endocrine neoplasms and to parallel progression of prostatic carcinoma (20, 21). Taken together, these results argue for the clinical usefulness of SgII or SgII-derived peptides in the diagnosis and/or prognosis of neuroendocrine tumors. The present study has shown that low tissue concentrations of EM66 are associated with malignant differentiation of pheochromocytoma, whereas benign pheochromocytomas contain about seven times higher quantities of EM66 peptide. It should be noted that the incidence of pheochromocytomas is only 2–8 cases per 1,000,000 subjects and that malignant pheochromocytomas only represent 10% of all pheochromocytomas. Hence, our investigation was performed on a relatively low number of patients. Larger numbers of benign and malignant pheochromocytomas should now be explored to confirm the difference in EM66 concentrations observed between the two types of tumors.

The concentration of EM66 found in our series of malignant tumors (0.48 pmol/mg protein) was in the same range as the concentration of SN reported by Eder *et al.* (18) in human pheochromocytomas. However, in this latter study, the authors did not address the question of the differentiation state of the tumor. The significantly higher levels of EM66 observed in benign *vs.* malignant pheochromocytomas may be ascribed to reduced proteolytic processing of SgII to EM66 in advanced stages of the tumor or by altered regulation of the secretion of the peptide. In line with these observations, it has been previously shown that serum levels of CgA increase with advanced tumor differentiation (29), whereas those of pancreastatin, a proteolytic CgA-derived fragment, decrease (30). Conversely, a direct correlation between serum SN levels and progression of prostate cancer has been reported (21). To determine whether EM66 can be used as a serum marker of benign *vs.* malignant pheochromocytomas, further studies are required to compare the levels of the peptide in the plasma and in tissue extracts from patients bearing the two types of tumors.

To date, the occurrence of metastases remains the sole criteria revealing malignancy of pheochromocytoma (31), so that the patients have a very poor survival rate when malignancy is diagnosed. In contrast, early detection and treatment of this type of tumor allow total remission in most cases. Several studies with controversial results have evaluated different parameters to predict malignancy of pheochromocytoma. Among these parameters are high preoperative 24-h urinary dopamine (immature secretion), extra-adrenal tumor location, high tumor weight, necrosis, MIB-1-positive cell rate (a proliferative index), depletion of S100-positive sustentacular cells, and increased CgA serum levels (32–36). In our study, univariate statistical analysis did not show any significant correlation between malignancy of the pheochromocytomas analyzed and sex, age, tumor location, or tumor diameter.

In conclusion, the present study suggests that measurement of the concentration of the SgII-derived peptide EM66 in pheochromocytomas may help to discriminate between benign and malignant tumors. Because, currently, malignancy of pheochromocytomas can only be diagnosed on the basis of the presence of metastastic lesions *i.e.* when the pronostic is very poor, EM66 may prove to be a valuable pronostic marker to predict the biological fate of pheochromocytomas and thus to improve the follow-up of the patients.

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† This work is dedicated to the memory of Dr. Vincent Contesse.

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