Peripherin, a Neuronal Intermediate Protein, Is Stably Expressed by Neuroendocrine Carcinomas of the Skin, Their Xenograft on Nude Mice, and the Corresponding Primary Cultures

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ABSTRACT

The histogenesis of neuroendocrine carcinomas of the skin is still controversial. To determine the degree of neural differentiation of these neoplasias, we studied the expression of intermediate filament proteins in tumoral tissues. Expressions of peripherin, the neurofilament protein NF-L, vimentin, and cytokeratin 8 were analyzed by immunohistochemical methods on 12 human primary tumors and 3 tumor xenografts on nude mice. Peripherin was detected in 10 primary tumors by immunofluorescence. The protein and the corresponding messenger RNA were identified by two-dimensional gel electrophoresis and Northern analysis in extracts of an immunofluorescence-negative tumor. Peripherin, NF-L, and cytokeratin 8 were detected in tumoral cells, whereas vimentin was found exclusively in the stroma. The histological and ultrastructural properties of the original cells of neuroendocrine carcinomas of the skin, as well as coexpression of peripherin, cytokeratin 8, and neurofilament polypeptides, were preserved in tumoral xenografts and their primary cultures in vitro. These results bring new elements to the knowledge of the biology of neuroendocrine carcinomas of the skin and indicate that peripherin constitutes a marker for tumor identification.

INTRODUCTION

NECs are a distinct entity of tumors also designated as Merkel cell tumors, because of similarities to the cutaneous neuroendocrine Merkel cells. These tumors, in addition to features characteristic of epithelial differentiation, express a program of neuroendocrine differentiation, including synthesis of dense-core neurosecretory granules, neuropeptide hormones, and neuron-specific enolase. Neverthe

MATERIALS AND METHODS

Tissues. Specimens of 12 cases of NECs (11 primary tumors and 1 local recurrence) were obtained by surgery. Small pieces from each sample were either immediately frozen in liquid nitrogen and stored at -80°C, fixed either in Bouin’s fixative or in 10% buffered formalin and embedded in paraffin for conventional light microscopy, or fixed in glutaraldehyde for electron microscopy. All tumors were analyzed by light microscope examination and indirect immunofluorescence (coexpression of cytokeratin, neurofilament proteins, and neuron specific enolase). Five tumors were examined by electron microscopy.

Nude Mice Xenografts. Five-7-week-old pathogen-free congenital athymic Swiss nude mice (Iffa-credo, les Oncins, France), were anesthetized with sodium pentobarbital (Nembutal). Biopsies of 3 primary NECs (M1, M2, M3), with an average size of 5 mm3, were implanted s.c. through an incision at the base of the tail. Animals were housed in a sterile environment and tumor growth was monitored twice a week. Tumors which had grown to 1-1.5 cm in diameter were subpassaged as for the first implantation. At each passage, xenografts were studied by light and electron microscopy.

NECs Cell Culture. Tumoral tissues were minced with scissors and cultured either on collagen-coated supports or on 3T3 feeder layers in Dulbecco’s modified Eagle’s medium (GIBCO) containing 10% fetal calf serum (GIBCO) and 5% inactivated horse serum (GIBCO). Cells were fed with fresh medium every other day and maintained in culture for at least 6 months.

Electron and Light Microscopy. Tumor specimens and smears of cultured NECs cells were immersed for 5 h at 4°C in 3% glutaraldehyde fixative in cacodylate buffer (pH 7.4) containing 5% sucrose and 0.03M CaCl2. After extensive washing in cacodylate buffer, samples were postfixed in 1% osmium tetroxide for 1 h at 20°C. Ultrathin sections were cut with a diamond knife in a Reichert Ultracut microscope, stained with uranyl acetate and lead citrate, and examined with a Jeol 1200 EX microscope. For light microscopy, semithin sections of specimens and smears of cultured cells were dehydrated in ethanol, embedded in Epon, and stained with hematoxylin-eosin.

Antibodies. The rabbit anti-peripherin polyclonal antibody was routinely used at a 1/100 dilution for immunohistochemistry and at a 1/5000 dilution for Western blotting. The specificity of this antibody was already documented.

The anti-NF-L neurofilament protein mAb (Sigma clone NR4) was used at a dilution of 1/30 for indirect immunofluorescence and at 1/200 for Western blotting. The anti-vimentin mAb (Sigma clone 13.2) was used for immuno-
histrochemistry at a 1/100 working dilution. The Troma 1 mAb, recognizing basic keratin 8, was a kind gift from Dr. Kemler and was used at a 1/30 dilution (15).

Fluorescein isothiocyanate-, tetramethyl isothiocyanate-, or rhodamine isothiocyanate-labeled anti-rabbit (1/30 final dilution), anti-rat (1/30 final dilution) or anti-mouse (1/50 final dilution) (DAKO) were the second antibodies for indirect immunofluorescence. Peroxidase-conjugated anti-mouse or anti-rabbit IgGs (1/100 final dilution; Diagnostic Pasteur) were the second antibodies for Western blotting.

**Indirect Immunofluorescence.** Drops of NECS cell suspensions were partially dried on glass slides and fixed in acetone for 10 min at 4°C. Five-µm-thick sections from frozen tissues were air dried and fixed in acetone as described previously (16). Samples washed 15 min at 4°C in PBS were incubated 1 h for 37°C with the primary antibody diluted in PBS (see "Antibodies"). After extensive washing in PBS, sections and smears were incubated with the appropriate secondary antibodies. Sections stained with a fluorescein isothiocyanate conjugate were counterstained with propidium iodide and mounted in ÁŒOVc glycerol-paraphenylene diamine. Slides were examined with a Zeiss Universal Microscope with epiillumination using an HBO 50 mercury arc lamp, III RS epifluorescence condenser, and fluorescein isothiocyanate or tetramethyl isothiocyanate filters. Photographs were taken on Kodak Ektachrome 400 film using a Zeiss MC 63 camera system.

**Polypeptide Analysis by Bidimensional Gel Electrophoresis and Western Blotting.** NECS tumoral tissues were homogenized and proteins of the crude extracts were separated by two-dimensional gel electrophoresis according to the method of O'Farrell (17) and modified as described (18). Gels were stained with Coomassie blue. Peptides were then blotted for 45 min onto nitrocellulose membranes (Hybond C; Amersham International UK) at 700 mA in a Transphor apparatus (Hoefer Scientific Instruments) as described (19). Peptides were first visualized on the nitrocellulose membrane by 5 min staining with Ponceau red (0.2% Ponceau red in 3% trichloroacetic acid), located with a marker, and then destained with distilled water.

**Northern Blot Analysis.** Total mRNAs were prepared from tumoral tissues according to the method of Chomczynski and Sacchi (20) and poly(A)+ mRNAs were purified following standard techniques.

Seven µg of poly(A)+ mRNA from NEC xenografts and 15 µg of poly(A)+ mRNA from primary tumors were fractionated by electrophoresis on 1.2% denaturing agarose gel containing 1.2 µM formaldehyde and transferred onto nylon membrane (Hybond N; Amersham). Membranes were subsequently hybridized in 50% formamide at 42°C with either peripherin or β-actin 32P-labeled random-primed cDNAs and exposed to hyperfilm MP (Amersham) with intensifying screens. rRNA and 0.24–9.5-kilobase RNA ladder (GIBCO-BRL, France) were used as molecular weight standards.

**RESULTS**

Upon light and electron microscopy examination, the 12 primary tumors presented the hallmarks of neuroendocrine skin carcinomas (1). All tumors were positively stained with the anti-cytokeratin 8 antibody and with an antibody raised against the neurofilament protein NF-L. The tumors reacted with the anti-vimentin antibody, but fluorescence was specific to the cells of the stroma. The anti-peripherin polyclonal antibody stained 10 of the 12 NECS (Fig. 1; Table 1). The antibodies reacted against cytoplasmic proteins: the anti-cytokeratin antibody clearly bound to thin cytoplasmic proteins, whereas the anti-peripherin and the anti-NF-L antibody did not define any organized network. All the three antibodies stained paranuclear balls in large tumor cells.

Table 1 Detection of intermediate filaments in human neuroendocrine carcinomas of the skin

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Primary tumors</th>
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<tr>
<td></td>
<td>Positive/total</td>
<td>Negative/total</td>
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<tr>
<td>Anti-cytokeratin 8 (monoclonal)</td>
<td>12/12</td>
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<tr>
<td>Anti-NF-L (monoclonal)</td>
<td>9/10</td>
<td>1/10</td>
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<tr>
<td>Anti-peripherin (polyclonal)</td>
<td>10/12</td>
<td>2/12</td>
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<tr>
<td>Anti-vimentin (monoclonal)</td>
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Fig. 1. Immunofluorescence microscopy of cutaneous neuroendocrine carcinoma cells using antibodies against intermediate filament proteins. Specimens were treated with antibodies directed against cytokeratin 8 (a), vimentin (b), peripherin (c), or NF-L protein (d). Note the specific labeling of tumor (a, c, d) and stroma cells (b). × 200.
In order to follow a possible phenotypic evolution of tumoral tissues during proliferation, tumor explants were xenografted on nude mice. Implants were passaged in vivo every 4 months, on average, for at least 3 years. Tumoral growth was slightly enhanced in animals grafted with explants from xenografts obtained after a high number of passages. Animals were sacrificed when the xenograft reached a size of 2 cm and the excised tumor tissues underwent macroscopic examination. Xenografts were always localized in the dermis, with no evidence of invasive proliferation. Neoplastic cells were arranged in solid sheets and cellular cords formation were faintly visible at the...
Table 2: Detection of peripherin expression in xenografted and primary NECS

<table>
<thead>
<tr>
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<th>M₁⁺</th>
<th>M₂⁺</th>
<th>M₃⁺</th>
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<tr>
<td>Indirect immunofluorescence</td>
<td>-</td>
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<td>Two-dimensional SDS/PAGE</td>
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<td>Western blotting</td>
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<td>Northern blotting</td>
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ᵃ NECS grafted on athymic nude mice.
ᵇ Primary NECS.
ᶜ SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

tumor periphery. At the microscopic level, the tumoral cells displayed a homogeneous shape and high rates of mitoses. As assessed by electron microscopy, the majority of the xenograft tumor cells retained the ultrastructural features typical of NECS, including membrane-bound dense-cored neuroendocrine-like granules, paranuclear whorl-like intermediate filament aggregates and occasional rudimentary junctions (Fig. 2). The general immunohistological picture drawn from studies performed on three tumor xenografts was similar to that previously obtained from the studies performed on the primary tumors. With the exception of the tumor xenograft M₁, which was negative for the expression of peripherin, the other two tumors were indistinguishable from the corresponding original tumors. Results are summarized in Tables 1 and 2.

To verify whether the phenotypic and biochemical properties of NECS cells were stably maintained in an artificial environment, primary cultures were established in vitro from tumor expiants. When cultured on plastic, all the tumor cells maintained a round shape and grew as loosely packed floating aggregates, amorphous and irregular in outline (Fig. 3). Indirect immunofluorescence performed on smears revealed that each cell expressed a pattern of cytoskeletal proteins similar to that observed in the corresponding tumors. Cells could be stained by the anti-peripherin, the anti-NF-L, and the anti-cytokeratin antibodies but not by the anti-vimentin antibody. Interestingly, and with the exception of the M₁ tumor-derived cultures, peripherin expression was stronger in cultured cells than in the original tumor cells. Double immunofluorescence labeling for peripherin and NF-L revealed a partial overlap of the cytoplasmic staining. However, the two types of intermediate filaments were constantly detected in the paranuclear balls, clearly conspicuous in large fractions of the cellular population (Fig. 3, c and d).

We then performed the biochemical characterization of NECS intermediate filament polypeptides. A primary NECS tumor (NECS-1) and two NECS xenografts (M₁-M₂) were analyzed by two-dimensional gel electrophoresis and subsequent immunostaining. Peripherin, NF-L, and cytokeratin 8 were clearly found coexpressed in NECS 1 and in the xenograft M₂ (Fig. 4, A, A' A' and C, C' C''). The amount of these two proteins was not stoichiometric, since in the xenograft M₂ NF-L was visualized as a small spot, whereas peripherin remained undetectable. Interestingly, the amount of vimentin found in the stroma was proportional to the amounts of peripherin and NF-L detected in tumoral tissues (Fig. 4). Although the immunofluorescence studies show that vimentin is expressed only in stroma, this protein was detected in all gels, this because of the unavoidable presence of stroma cells in the tumor samples. An important observation is that both peripherin and vimentin migrated at a pH, lower than that expected (9). The cause for this altered migration is under investigation.

Lack of immunological staining cannot be regarded as an absolute criterion for excluding the possible synthesis of low amounts of peripherin. We therefore examined on xenografts whether absence of immunological staining of peripherin could be correlated with the absence of specific RNA transcripts. Poly(A)⁺ mRNAs were prepared from a primary tumor (NECS-1), from the xenograft M₂, and from the
NEUROENDOCRINE CARCINOMAS OF THE SKIN EXPRESS PERIPHERIN

Fig. 4. Identification by two-dimensional gel electrophoresis and Western blotting of cytoskeletal components of NECS cells. Portions of Coomassie blue-stained two-dimensional gels loaded with an extract of a primary tumor (NEC-1) (A), or with extracts from the NECS grafted on athymic nude mice M1 (B), and M2 (C). Gels are shown before (A-C) and after Western blotting on a nitrocellulose membrane and immunodetection with the monoclonal antibody against NF-L (A’, B’, C’), the polyclonal against peripherin (A’”, B’”, C’”) and the monoclonal against vimentin (A’””, B’””, C’””). NF-L, low-molecular-mass neurofilament protein; Pe, peripherin; Vi, vimentin; ViD, vimentin-derived peptides; α, β, corresponding subunits of tubulin. The slightly decorated spots on the left side of A’”” and C’”” are due to degradation products of lamins.

xenograft M1, negative for this protein. The probe was a peripherin cDNA clone isolated from a murine neuroblastoma Agt 11 cDNA library by immunoscreening (10). As shown in Fig. 5, hybridization of Northern blots with the [32P]cDNA revealed the presence of a specific 2-kilobase transcript both in the xenograft M1 and, as expected, in the peripherin-positive tumors M2 and NECS 1. Surprisingly the amount of peripherin transcripts in M1 was estimated to be at least 3 times higher than that detected in M2. The different rates of transcription in the xenografts were confirmed by rehybridization of the blot with a β-actin probe. In contrast to the xenograft M2, the M1 tumor exhibited higher levels of transcription for peripherin than for β-actin. The intensity of the hybridization bands was comparable in the NECS 1 preparation.

DISCUSSION

In this report we demonstrate that NECS cells express peripherin. Synthesis of the protein was first monitored in human tumors by indirect immunofluorescence using a specific antibody that does not cross-react with other cytoskeletal components (9). A strong labeling of tumor cells indicated the presence of peripherin (Fig. 1a) coexpressed with cytokeratins and the small neurofilament NF-L protein (Fig. 3c and d). This observation reveals the complexity of the cytoskeletal structures of NECS, which display patterns common both to simple and stratified epithelia and to neural cells. Cytokeratin and NF-L neurofilament protein appear as thin cytoplasmic fibrils and perinuclear plaques. Accordingly to previous observations (6, 16, 21), perinuclear plaques were brightly stained by anti-cytokeratin and antineurofilament antibodies, whereas fibrils reacted predominantly with anti-cytokeratin antisera. We could not determine by double labeling techniques whether cytokeratin and neurofilament proteins are found within the same intermediate filaments or whether peripherin filaments and neurofilaments are distinct but closely intermingled. It is also not clear whether peripherin is a constituent of NECS heteropolymer filaments, or whether it provides a distinct filament structure. The anti-peripherin antibody strongly stained the perinuclear plaques, thus suggesting the presence of peripherin in the aggregates. The coexpression of the three different types of intermediate filament proteins was confirmed by double immunofluorescence experiments. The nature of the paranuclear aggregates, alternatively termed fibrous bodies or perinuclear aggregates, is unknown. Ultrastructurally, they correspond to whorl-like intermediate filaments and could arise from a depolymerization of the intermediate filament network possibly caused by overphosphorylation of cytoskeletal components. In-
Endocrine cells express neural markers that are detected at key pig snout, suggesting that intraepidermal Merkel cells express peripheral proteins. The expression of peripherin by normal human Merkel cells are lacking, and the amino-terminal domain of NF-L induce a depolymerization of intermediate filaments in vitro (22–24). Moreover, altered phosphorylation patterns of vimentin and peripherin would be compatible with the more acidic pI of these proteins extracted from NECS cells seen by isoelectric focusing (Fig. 4).

The results obtained on NECS cells were confirmed by the analysis of xenografted tumors. Even after a high number of serial heterotransplantations, xenografts were morphologically indistinguishable from the original tumors, as evidenced by the presence of neurosecretory granules, neuron-specific enolase, and neuropeptides (data not shown). The histological and ultrastructural properties of the original NECS cells were also preserved in NECS cells derived from primary cultures of xenografted tumors upon expansion in vitro. Coexpression of peripherin, cytokeratins, and neurofilament polypeptides was maintained at elevated rates, while synthesis of vimentin remained undetectable. In culture, NECS cells adhered poorly to plastic substrates and showed average mean doubling times exceeding 5 days. Attempts to improve adhesion by coating dishes and seeding cells on fibroblast feeder layers were unsuccessful, as well as addition of mitogenic factors to media (epidermal growth factor, neuronal growth factor, basic fibroblast growth factor, from horse serum) to stimulate growth. Similar observations had been previously made on the neuroendocrine skin carcinoma cell line MKL-1 (25) and on primary cultures of a lymph node metastasis (26).

Injection of NECS cells induced tumors in mice after latency periods of 3–5 months, without occurrence of detectable metastasis. The histological and ultrastructural characteristics of NECS found again in all the analyzed tumors gave evidence of the stability of the phenotype (not shown).

The biological role of peripherin is still unclear. The protein may play a role in the recognition of the axonal pathway (27, 28) and its activation of suppressed functions. In adult chromaffin cells and β-cells of Langerhans islets and in the corresponding tumors, neural-specific genes are inactive. However, reactivation of peripherin in NF-L filament protein synthesis in the tumor-derived pheochromocytoma PC12 cell line and in the insulinoma RINSF cells was demonstrated (14). Moreover, in primary cultures of normal endocrine cells, expression of neural intermediate filaments appears to be correlated with the absence of intercellular interactions preventing extending processes (31, 32).

Peripherin constitutes an additional marker for the identification of the histogenesis of NECS. Of 12 tumors, 10 were positive for immunostaining with a peripherin-specific antibody. Moreover, when further analyzed by Northern blot, an apparently negative tumor displayed a low level of expression of peripherin mRNA. In this context, the relevance of peripherin as a marker for differential diagnosis, e.g., to discriminate between primary NECS and secondary location of neuroendocrine tumors of extraneous origin is still to be evaluated. It is well established that rat pheochromocytoma and mouse neuroblastoma cells express peripherin, however, no data are yet available from other human neuroendocrine tumors of epithelial type, such as pulmonary neuroendocrine tumors, the medullary carcinoma of the thyroid, islet cell tumors of pancreas, and the carcinoid of small intestine. Further investigations on the distribution of peripherin in a wide variety of neuroendocrine cells and related tumors are therefore required.

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REFERENCES

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