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Aberrant expression of receptors for platelet-derived growth factor in an anaplastic thyroid carcinoma cell line

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ABSTRACT **Receptors for platelet-derived growth factor (PDGF) have previously only been found on cells of mesenchymal and glial origin. This study shows PDGF receptors on an anaplastic thyroid carcinoma cell line, C 643, that was found to express thyroglobulin mRNA, confirming its origin from thyroid epithelium. Northern blot analysis of poly(A)$^+$ RNA hybridized with a human PDGF B-type receptor cDNA probe revealed a 5.4-kilobase transcript in the C 643 cells. The existence of receptor protein on the cell surface was shown by immunofluorescence microscopy with a PDGF receptor monoclonal antibody. Binding experiments with ^{125}I-labeled dimeric forms of PDGF indicated that the cells contain B-, but not A-, type PDGF receptors. The addition of PDGF to C 643 membranes in the presence of [^{32}P]ATP induced phosphorylation of the receptor. A polyclonal PDGF B-type receptor peptide antiserum was used to immunoprecipitate a receptor protein from metabolically labeled C 643 cells; the receptor was found to be 5–10 kDa larger than that in normal human fibroblasts. Removal of N-linked carbohydrates using endoglycosidase H resulted in deglycosylated receptor proteins of similar size in C 643 cells and fibroblasts, indicating differences in glycosylation patterns of the two receptor proteins. The aberrant expression of receptors might be crucial in tumor development by conferring a selective growth advantage to the cancer cells.**

Recent studies of oncogenes in relation to the structure and function of polypeptide growth factors have yielded a unifying concept of neoplastic transformation and normal growth stimulation. This view is based on the finding that oncogenes represent altered versions of normal cellular genes (protooncogenes) (1), which encode proteins that operate along the mitogenic pathway (2). Oncogenes are therefore supposed to transform the cell by generating an abnormal growth stimulus, which causes a short-circuit in the mitogenic pathway. Thus, an unscheduled synthesis of a growth factor in cells that carry the cognate receptor (autocrine growth stimulation) is generally thought to be one of several mechanisms of transformation (2, 3). The most convincing evidence for this view has been derived from studies on the structure of platelet-derived growth factor (PDGF), which showed a virtual identity between the PDGF B chain and part of the oncogene product (sis) of simian sarcoma virus (SSV) (4, 5); SSV transformation has been found to be mediated by an autocrine PDGF-like growth factor (reviewed in ref. 6).

PDGF consists of dimers of A and B chains linked by disulfide bridges (7). All three possible dimeric forms have been identified: PDGF-AB (present in human platelets; ref. 8), PDGF-AA (isolated from conditioned medium of human tumor cells; refs. 9 and 10), and PDGF-BB [present in human (8) and porcine (11) platelets, as well as the transforming gene product of SSV (ref. 12)]. The three dimers interact with different specificities to two distinct receptor species (types A and B) on fibroblasts (13, 14). The B-type receptor is identical to the previously identified PDGF receptor with protein tyrosine kinase activity (reviewed in ref. 15), which has been molecularly cloned (16, 17). All PDGF dimers bind to the A-type receptor, whereas the B-type receptor binds PDGF-BB and PDGF-AB only, the latter, however, with lower affinity.

Expression of PDGF receptors has been found to be restricted to cells of mesenchymal and glial origin (18), whereas various types of epithelial cells, including thyroid follicle cells, lack PDGF receptors and do not respond to the factor. This study reveals an apparently structurally altered PDGF B-type receptor in human epithelial-derived cells; namely, the anaplastic thyroid carcinoma cell line C 643. That this cell line is derived from bona fide thyroid follicle cells was shown by the expression of thyroglobulin mRNA. The expression of PDGF receptors in epithelial cells may be of functional significance in transformation and carcinogenesis.

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions. The cell line C 643 was established from a fine-needle biopsy of an anaplastic thyroid carcinoma of a 76-year-old man (19). The patient died within 5 months after diagnosis. Karyotype analysis revealed severe chromosomal abnormalities, including an increased number of chromosomes and the occurrence of marker chromosomes. The C 643 cell line and a normal human fibroblast cell line AG 1523 (obtained from the Human Genetic Cell Mutant Repository, Camden, NJ) were cultured in Eagle's medium supplemented with antibiotics (100 units of penicillin and 50 μg of streptomycin per ml) in the presence of 10% newborn calf serum).

PDGF. PDGF was purified from human platelets (20), and consists of ≈70% PDGF-AB and 30% PDGF-BB (8). PDGF-AA and -BB homodimers used in the binding experiments were purified from supernatants of yeast cells expressing PDGF-AA and -BB, respectively (A. Östman, G. Bäckström, N. Fong, C. Betsholtz, V. Hellman, B.W., P. Valenzuela, and C.-H.H., unpublished data). PDGF-AA and -AB were labeled according to the chloramine-T method (21) (specific activity, 40,000 cpm/ng) and PDGF-BB was labeled by the Bolton–Hunter method (22) (specific activity, 70,000 cpm/ng).

RNA Extraction. RNA was extracted from the cells as described (23). The cells were homogenized in 6 M urea/3 M LiCl/0.2% sodium dodecyl sulfate (SDS)/Antifoam A (1 μl/ml) (Sigma; A 5758), and left on ice overnight. The

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Abbreviations: PDGF, platelet-derived growth factor; ^{125}I-PDGF, ^{125}I-labeled PDGF; BSA, bovine serum albumin; EGF, epidermal growth factor.

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precipitate was centrifuged at 16,000 × *g* for 20 min and the pellet was dissolved in 10 mM triethanolamine, pH 7.5/1 mM EDTA/0.5% SDS. The RNA was extracted once with phenol and then with chloroform/isoamyl alcohol (24:1) and precipitated by addition of 0.1 vol of 3 M sodium acetate and 2.2 vol of ethanol. Before electrophoresis, the RNA samples were poly(A)$^+$-selected on an oligo(dT)-Sepharose column and quantitated spectrophotometrically.

Northern Blot Hybridization. Poly(A)$^+$ RNA samples (5 μg per lane) were electrophoresed on a formaldehyde/agarose gel [0.8% agarose/2.2 M formaldehyde/2× Northern runner (50× stock solution: 1 M Mops, pH 7.0/50 mM EDTA/0.25 M NaOAc] and transferred to a nitrocellulose filter by a blotting procedure. The filter was prehybridized for 24 hr at 42°C in a buffer consisting of 50% formamide/5× SSC (1× SSC = 0.15 M NaCl/0.0125 M sodium citrate, pH 7.0)/1× Denhardt's solution [0.02% bovine serum albumin (BSA)/0.02% Ficoll/0.02% polyvinylpyrrolidone]/5 mM NaH_2PO_4/5 mM Na_2HPO_4/0.1% SDS/salmon sperm DNA (200 μg/ml), followed by hybridization in the same buffer containing [^{32}P]DNA probes (labeled with Multiprime labeling kit, Amersham; ≈10^9 cpm/μg of DNA). After a 24-hr hybridization (42°C) the filter was washed for 2 hr in 2× SSC/0.5% SDS at 65°C and, if necessary, for 30 min in 0.1× SSC/0.1% SDS. The filter was autoradiographed at −70°C with Kodak XAR-5 film and intensifying screens (Du Pont).

The amount of RNA per lane was controlled by hybridization of the filter to a human glyceraldehyde-3-phosphate dehydrogenase cDNA probe [pHcGAP$_3$, kindly provided by Ray Wu, Cornell University (24)]. The human thyroglobulin cDNA probe (phTg1) was kindly provided by Gilbert Vassart (Université Libre de Bruxelles, Belgium) (25).

Immunofluorescence Microscopy. A mouse monoclonal antibody (PDGFR-B2) was used for immunofluorescence microscopy of the PDGF receptors (26). Cells growing on coverslips were incubated with antibodies (5 μg/ml) for 30 min at room temperature, washed in phosphate-buffered saline (PBS), and fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody was added for 30 min. The cells were fixed in 3% paraformaldehyde in PBS, mounted on microscope slides, and photographed in a Leitz UV microscope equipped for epifluorescence. A mouse monoclonal antibody (C2, a collagen type II antibody) of the same isotype (IgG2b) was used as a control.

PDGF Binding Assay. Confluent cultures of cells in 12-well dishes (Costar) were used for ^{125}I-labeled PDGF (^{125}I-PDGF) binding in 0.5 ml of PBS supplemented with 1 mg of BSA per ml. Radiolabeled PDGF isoforms (^{125}I-PDGF-AA, ^{125}I-PDGF-AB, and ^{125}I-PDGF-BB; ≈5 × 10^4 cpm per well) were added in the presence or absence of unlabeled PDGF-AA or PDGF purified from human platelets (50 ng/ml). After a 2-hr incubation on ice, the cells were washed five times with ice-cold PBS complemented with 1 mg of BSA per ml. Cell-associated radioactivity was solubilized with 1% Triton X-100 and counted in a γ-counter (Beckman; Gamma 8000).

Autophosphorylation Assay. Membranes were prepared as described (27). The phosphorylation reaction was carried out at 0°C in a final vol of 40 μl containing 10 μg of C 643 membranes, 3 mM $MnCl_2$, 25 μM Na_3VO_4 in 20 mM Hepes (pH 7.4) (28). After a 10-min incubation with or without 100 ng of PDGF purified from human platelets, 15 μM ATP containing 5 × 10^6 cpm of [γ-^{32}P]ATP was added and incubated for another 10 min before the reaction was stopped by the addition of 50 μl of SDS sample buffer (80 mM Tris·HCl, pH 8.8/3.6% SDS/10 mM dithiothreitol/3.8% sucrose/0.01% bromophenol blue). The samples were heated at 95°C for 3 min and alkylated with 50 mM iodoacetamide before SDS gel electrophoresis (polyacrylamide gradient of 5–10%) (29).

Immunoprecipitation and Endoglycosidase H Sensitivity of the PDGF Receptor. The C 643 cells were metabolically labeled with a mixture of [^{35}S]cysteine and [^{35}S]methionine (each at 100 μCi/ml) (Amersham; specific activity, >800 and >600 Ci/mmol, respectively; 1 Ci = 37 GBq) for 3 hr in cysteine- and methionine-free Eagle's minimum essential medium supplemented with 10% dialyzed fetal calf serum. The immunoprecipitation procedure used two different rabbit polyclonal PDGF receptor antisera; PDGFR-1, recognizing both A- and B-type receptors, and PDGFR-3, recognizing only the B-type receptor (43). PDGFR-1 was raised against purified porcine receptors (30) and PDGFR-3 was raised against a 14-amino acid synthetic peptide corresponding to amino acids 981–994 in the deduced sequence of the murine B-type receptor (16). The immunoprecipitations were performed in sequence starting with the PDGFR-3 antiserum followed by the PDGFR-1 antiserum. The precipitates were incubated in the presence and absence of endoglycosidase H (New England Nuclear), which removes N-linked carbohydrates in the high-mannose form (31). The samples were analyzed by 5–10% gradient SDS gel electrophoresis (29) followed by autoradiography.

RESULTS

Properties of the C 643 Cell Line. Microscopic analysis of fixed and stained cultures of the anaplastic thyroid carcinoma C 643 cell line showed a marked cellular atypia with the presence of multilobulated nuclei and giant nuclei. Despite the undifferentiated morphology of the cell line, it retained the expression of the thyroglobulin gene as a marker of thyroid follicle cell function. Thus, Northern blot hybridization using a human thyroglobulin cDNA probe revealed 8.4-kilobase (kb) thyroglobulin transcripts in C 643 cells (Fig. 1). A similar RNA species was present in normal thyrocytes (data not shown), but not in normal human fibroblasts (Fig. 1).

Expression of PDGF Receptor mRNA. Northern blot hybridization using a human PDGF B-type receptor cDNA probe on poly(A)$^+$ RNA from C 643 cells under stringent conditions showed the expression of PDGF B-type receptor transcripts of the same size (5.4 kb) as in normal human fibroblasts, AG 1523 (Fig. 2). The probe did not hybridize to RNA extracted from another anaplastic thyroid carcinoma lacking PDGF receptors (N.-E.H. and B.W., unpublished observation). However, the amount of mRNA in the C 643 cells was lower than in AG 1523 cells.

Binding of PDGF. Since PDGF receptor transcripts were found in C 643 cells it was of interest to determine whether a receptor with normal ligand binding properties was synthesized. Therefore, a binding assay using the various radiolabeled dimeric forms of PDGF was performed. As shown in Fig. 3, the anaplastic thyroid carcinoma cells bound ^{125}I-PDGF-AB and -BB, but not ^{125}I-PDGF-AA. Unlabeled PDGF-AA did not compete with the binding of ^{125}I-

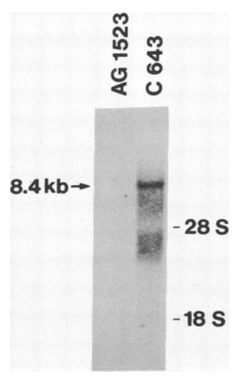

FIG. 1. Expression of thyroglobulin mRNA in C 643 cells. Total RNA was extracted from cells, poly(A)$^+$ enriched, electrophoresed, blotted, and hybridized, and subjected to autoradiography. The filter was hybridized with a ^{32}P-labeled 1.6-kb *Pst* I fragment of the human thyroglobulin cDNA phTg1 (25).

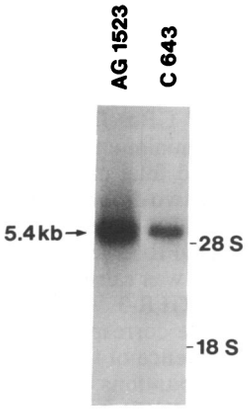

FIG. 2. Expression of PDGF receptor mRNA in C 643 cells. Total RNA was extracted from C 643 and AG 1523 cells and subjected to poly(A)$^+$ selection. Samples (5 μg/ml) of poly(A)$^+$ RNA were electrophoresed and blotted and subjected to autoradiography. The filter was hybridized to a ^{32}P-labeled 2.8-kb human PDGF B-type receptor cDNA probe (17).

PDGF-AB or -BB. The C 643 cell line was found to express and synthesize PDGF-AA (data not shown); it is therefore possible that the lack of PDGF-AA binding was due to down-regulation caused by endogenous PDGF-AA. An experiment was therefore performed with suramin, an agent that prevents the interaction between PDGF and its receptor(s) (32), thereby causing up-regulation of receptors in cells expressing both receptor and the corresponding ligand. Incubation of C 643 cells for 24 hr at 37°C in 200 μg of suramin per ml did not increase the binding of ^{125}I-PDGF-AA (data not shown). Taken together, these data indicate the presence of type B but not A PDGF receptors on the C 643 cells.

Visualization of Cell-Surface PDGF Receptors by Immunofluorescence. The PDGF receptors on C 643 cells could be visualized by an indirect immunofluorescence microscopy technique using a mouse monoclonal PDGF B-type receptor antibody (PDGFR-B2). Fig. 4*A* shows positive staining on C 643 cells, as well as on the human fibroblasts used as a positive control (Fig. 4*D*). Fig. 4*B* shows the lack of staining of the C 643 cells using a control mouse monoclonal antibody of the same isotype. Prior treatment of C 643 cells with PDGF (100 ng/ml) resulted in disappearance of the receptors from the cell surface (Fig. 4*C*), which indicates normal internalization of the receptor.

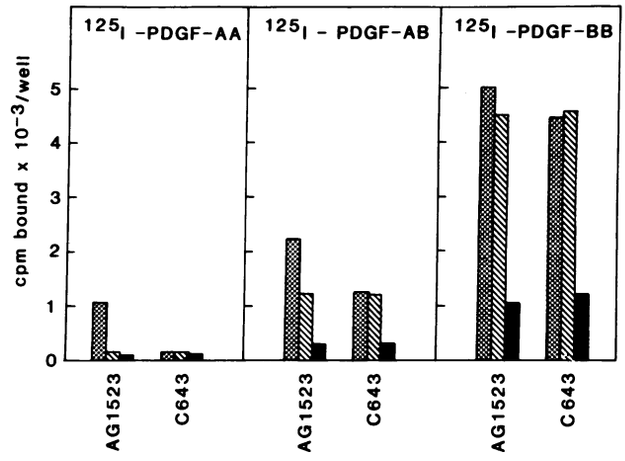

FIG. 3. Binding of different dimeric forms of PDGF. Binding of PDGF to C 643 cells was investigated using radiolabeled dimeric forms of PDGF. Unlabeled PDGF-AA (▨) and PDGF purified from human platelets (■) (50 ng/ml) were added to compete with the radiolabeled ligands for binding. A normal human fibroblast cell line AG 1523 was used as a control (▧).

Autophosphorylation of the PDGF Receptor in C 643 Cells. Addition of PDGF to fibroblasts induces a characteristic autophosphorylation of the PDGF receptor (28). As shown in Fig. 5, the addition of PDGF to C 643 membranes induced phosphorylation of a 185-kDa component, most likely representing autophosphorylation of the receptor. This receptor appeared to be significantly larger than the PDGF receptor phosphorylated in normal human fibroblasts (180 kDa) (data not shown).

Immunoprecipitation and Endoglycosidase H Digestion of the PDGF Receptor in C 643 Cells. Metabolically labeled C 643 cells were sequentially immunoprecipitated with two different polyclonal rabbit anti-PDGF receptor antisera: PDGFR-3 (precipitating only the B-type receptor) followed by PDGFR-1 (precipitating both A- and B-type receptors). A 185-kDa receptor protein was recognized by the PDGFR-3 antiserum, as well as a possible precursor protein of 160 kDa,

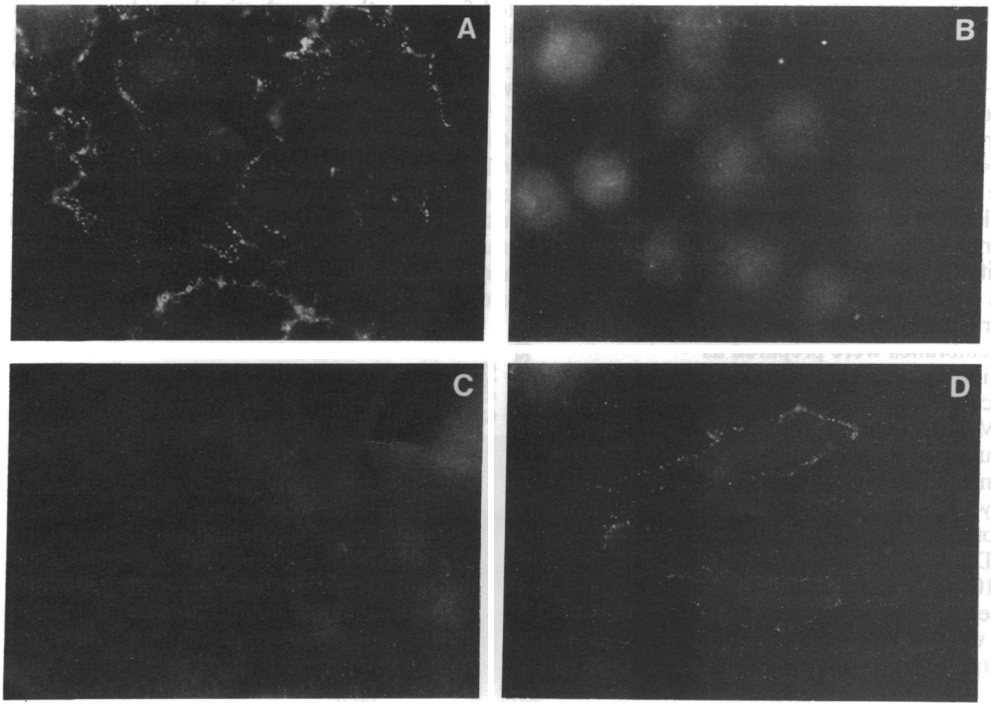

FIG. 4. Immunofluorescence microscopy. A mouse monoclonal antibody (PDGFR-B2) was used together with a fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody. The cells were photographed in a Leitz UV microscope equipped for epifluorescence (Kodak Tri-X film). (*A*) C 643 cells incubated with PDGFR-B2 antibodies; (*B*) C 643 incubated cells with C2 control antibodies (collagen type II antibody, isotype IgG2b); (*C*) C 643 cells preincubated 1 hr with PDGF (100 ng/ml) and thereafter incubated with PDGFR-B2 antibodies; (*D*) AG 1523 fibroblasts incubated with the PDGFR-B2 mouse monoclonal PDGF receptor antibody, as a positive control.

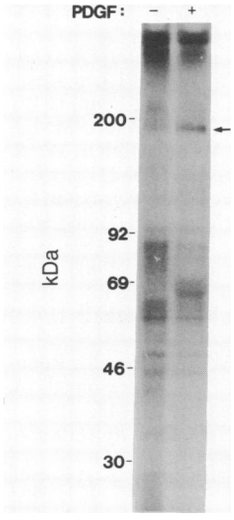

FIG. 5. Autophosphorylation of the PDGF receptors present in C 643 cells. Membranes from C 643 cells were incubated with or without 100 ng of PDGF in the presence of [^{32}P]ATP. Samples were analyzed by SDS gel electrophoresis under reducing conditions, followed by autoradiography. Arrow indicates the phosphorylated receptor protein.

in the C 643 cells (Fig. 6). Subsequent immunoprecipitation with PDGFR-1 antibodies brought down the 170-kDa A-type receptor in normal human fibroblasts (AG 1523). However, in C 643 cells, the antiserum did not recognize a 170-kDa component. The 110-kDa species that appeared in this immunoprecipitation was present in many cell lines and did not correlate to presence of A- or B-type receptors. The mature receptor protein found in C 643 cells appeared to be slightly larger (5–10 kDa) compared to the mature receptor of human fibroblasts (31), judged from its migration in SDS gel electrophoresis (Fig. 6). To examine whether the increased size of the C 643 cell-derived PDGF receptors was due to an increased size of the polypeptide or to altered posttranscriptional modification(s)—i.e., glycosylation—we incubated immunoprecipitated material in the presence or absence of

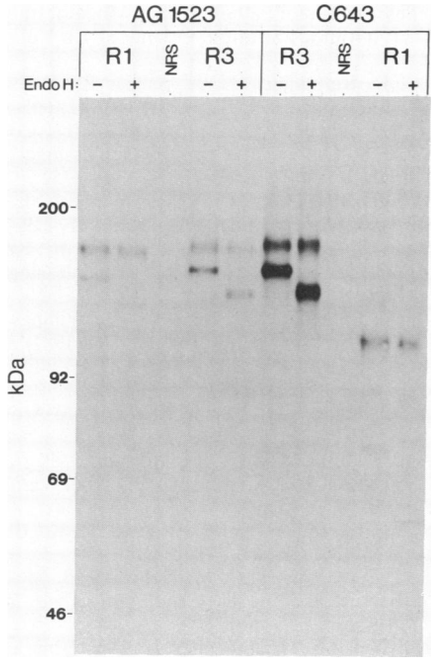

FIG. 6. Immunoprecipitation and endoglycosidase H digestion of the PDGF receptors. The C 643 and AG 1523 cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine and sequentially immunoprecipitated with two polyclonal rabbit antisera starting with PDGFR-3 followed by PDGFR-1. The precipitates were incubated with or without endoglycosidase H. A nonimmune rabbit serum (NRS) was used as control.

endoglycosidase H. This enzyme removes N-linked carbohydrates in the high mannose form. As shown in Fig. 6, the shift in molecular mass of the endoglycosidase H-sensitive component was larger in the C 643 cell-derived samples than in the samples derived from AG 1523 fibroblasts, yielding a similar migration rate for the two deglycosylated receptors. This finding supports the assumption that the PDGF receptors of C 643 cells and human fibroblasts differ in glycosylation.

DISCUSSION

This study provides evidence for aberrant expression of PDGF receptors in a human anaplastic thyroid cell line (C 643). The cells were found to express PDGF B-type receptor mRNA and contain specific ^{125}I-PDGF binding sites, as well as cell-surface receptor protein demonstrated by indirect immunofluorescence using mouse monoclonal antibodies. This is remarkable given that PDGF receptors have previously only been found on connective tissue- and glia-derived cells. Since anaplastic carcinoma of the thyroid, because of its undifferentiated character, might simulate other malignancies, including soft tissue sarcomas (33), it might be argued that the tumor did not originate from thyroid epithelium proper but had evolved from a mesenchymal element of the thyroid stroma. This is, however, unlikely since the C 643 cells were found to synthesize thyroglobulin mRNA.

The existence of two classes of receptors for PDGF (types A and B) has recently been demonstrated in normal human fibroblasts (13, 14). Their sizes were estimated to be 170 to 180 kDa, respectively (43). The PDGF receptor found in C 643 cells was shown to be B type, since the cells bound ^{125}I-PDGF-AB and -BB, but not ^{125}I-PDGF-AA. In this regard, the C 643 cells differ from human fibroblasts, which display both types of receptors. It is notable that experiments on fibroblasts have indicated that B-type receptors have a major role in mediating the mitogenic response to PDGF (13).

The PDGF B-type receptor is an integral membrane protein with an extracellular ligand binding domain, a transmembrane part, and an intracellular domain endowed with a tyrosine protein kinase, which is activated by ligand binding (15). The functional activity of the receptor on anaplastic thyroid carcinoma cells was demonstrated by its autophosphorylation when exposed to PDGF. Interestingly, the PDGF receptor present on anaplastic thyroid carcinoma cells was found to display a somewhat slower migration in SDS gel electrophoresis than PDGF receptors from human fibroblasts. The altered size of the thyroid carcinoma receptor is most likely explained by an abnormal glycosylation, since digestion with endoglycosidase H to remove N-linked carbohydrates revealed a normal size of the polypeptide backbone. The effect of this increased glycosylation on the functional activity of the receptor remains to be elucidated. Furthermore, normal internalization and down-regulation of the receptors were observed after stimulation of C 643 cells with PDGF.

Although a transforming potential of the PDGF B-type receptor has not been formally proven, its structural and functional homology with the tyrosine protein kinase family of oncogenes leads one to suspect that overexpression or structural alteration of the receptor protein may confer a transformed phenotype to the cell. Molecular cloning of mouse PDGF receptor cDNA (16) has revealed a close structural similarity with the *fms* and *kit* oncogenes. The normal homolog of *fms* encodes the receptor for macrophage colony-stimulating factor (34), whereas the cellular counterpart of *kit* is assumed to encode a receptor of an as yet unidentified growth factor (35).

At least five mechanisms have been identified whereby growth factor receptors are directly involved in transforma-

tion and tumorigenesis: overexpression of a normal receptor, gene fusion, point mutation, truncation, or autocrine receptor activation (combinations of these mechanisms also exist) (see ref. 36 for a recent review). Overexpression of epidermal growth factor (EGF) receptors has been observed in several human malignancies, notably in glioblastoma multiforme where at least a third of all cases have been shown to have an amplified EGF receptor gene (37). Direct evidence for the involvement of EGF receptor overexpression in tumorigenesis was recently obtained using NIH 3T3 cells transfected with a normal human EGF receptor cDNA (38, 39); tumorigenesis of these cells was enhanced by the administration of EGF to the transplanted animal (38). Conceivably, the aberrant expression of a mesenchymal growth factor receptor in epithelial cells may similarly confer a selective growth advantage and operate at a certain stage in tumor development. It is interesting to note that C 643 cells express PDGF A-chain mRNA and synthesize the corresponding protein (N.-E.H. and B.W., unpublished observation); this growth factor production has no significance for an autocrine response since the B-type receptor does not recognize PDGF-AA (13). Activation of the PDGF receptors on C 643 cells thus has to be mediated by exogenous B-chain containing isoforms of PDGF. *In vivo*, such ligands may be produced by normal cells present in the tumor, such as macrophages (40, 41) and endothelial cells (42).

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