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Production of platelet-derived endothelial cell growth factor by normal and transformed human cells in culture

KENSUKE USUKI*, NILS-ERIK HELDIN†, KOHEI MIYAZONO*‡, FUYUKI ISHIKAWA‡, FUMIMARO TAKAKU‡, BENGT WESTERMARK†, AND CARL-HENRIK HELDIN*§

*Ludwig Institute for Cancer Research, Biomedical Center, Box 595, S-751 23 Uppsala, Sweden; †Department of Pathology, University Hospital, S-751 85 Uppsala, Sweden; and ‡The Third Department of Internal Medicine, University of Tokyo, Hongo, Tokyo 113, Japan

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ABSTRACT **Platelet-derived endothelial cell growth factor (PD-ECGF) is a 45-kDa endothelial cell mitogen which has angiogenic properties *in vivo*. We report here that human foreskin fibroblasts, a human squamous cell carcinoma cell line, and 2 out of the 3 human thyroid carcinoma cell lines investigated produce PD-ECGF, whereas 21 other cell lines examined do not. The positive cell lines contained a 1.8-kilobase PD-ECGF mRNA, and a 45-kDa protein could be demonstrated in lysates of the cell lines by immunoblotting and immunoprecipitation using a specific antiserum against PD-ECGF. Furthermore, the cell lysates contained mitogenic activity for endothelial cells that was neutralized by the PD-ECGF antiserum. PD-ECGF was found to be secreted only slowly from the producer cells, consistent with the previous finding that the primary translation product lacks a signal sequence. The restricted expression and intracellular sequestration of PD-ECGF imply a strictly controlled function in endothelial cell proliferation and angiogenesis. Aberrant production of PD-ECGF may play a role in tumor angiogenesis.**

Platelet-derived endothelial cell growth factor (PD-ECGF; for a review see ref. 1) stimulates growth and chemotaxis of endothelial cells *in vitro* and has angiogenic activity *in vivo* (2, 3). It is a 45-kDa protein which, in contrast to angiogenic factors of the fibroblast growth factor (FGF) family (4), does not stimulate the growth of fibroblasts. PD-ECGF has been purified from human platelet lysate and seems to be the only endothelial cell mitogen in platelets (5). cDNA for PD-ECGF was recently cloned (3); the predicted amino acid sequence has no striking homology with that of previously known proteins. Interestingly, the primary translational product of PD-ECGF was found to lack a hydrophobic signal sequence, similar to what has been found for acidic (6) and basic (7) FGF.

Only limited information is available about the biosynthesis, release, and tissue distribution of this novel growth factor. As an initial step to answer these questions, we have studied the synthesis of PD-ECGF by different human cell lines. We report here that PD-ECGF is expressed in human foreskin fibroblasts, in a human squamous cell carcinoma cell line, and in two of three anaplastic thyroid carcinoma cell lines, but not in a number of other cell lines.

MATERIALS AND METHODS

Cell Lines. The following human cell lines were analyzed for PD-ECGF expression: the foreskin fibroblast cell line AG 1523 (obtained from the Human Mutant Cell Repository, Camden, NJ); the carcinoma cell lines HTh-7, C 643, and SW 1736, which originate from tumors diagnosed as anaplastic thyroid carcinoma (8); the cell lines U-1240 MG, U-410 MG, U-178 MG, U-251 MG, U-343 MG, U-343 MGa Cl2:6, and U-343 MGa Cl35L, which derive from malignant glioma (9); the osteosarcoma cell line U-2 OS (10); the cell lines U-2197 and U-2149, which were established from malignant fibrous histiocytoma (obtained from M. Nistér, Uppsala); the squamous cell carcinoma line A 431 (obtained from J. De Larco, National Institutes of Health, Bethesda, MD); the HEP G2 hepatoma cell line (obtained from J. Scott, Medical Research Council, Harrow, Middlesex, U.K.); the GS kidney adenocarcinoma cell line (obtained from V. Sovova, Institute of Molecular Genetics, Prague), the T 24 bladder carcinoma cell line (obtained from P. Perlman, University of Stockholm); the colon carcinoma cell lines Colo 320 and SW 1116 (obtained from the American Type Culture Collection, Rockville, MD); the melanoma cell line WM115 (obtained from M. Herlyn, Wistar Institute, Philadelphia); the megakaryocytic leukemia cell line CMK (obtained from H. Nakajima, Chiba, Japan); and the erythroleukemia cell lines HEL and K562 (obtained from the American Type Culture Collection and K. Nilsson, Uppsala, respectively). Normal thyroid epithelial cells were isolated from porcine thyroid glands obtained from a local slaughterhouse. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 medium containing 10% fetal calf serum (FCS) and antibiotics at 37°C in a humidified atmosphere containing 5% CO_2.

Immunoblot Analysis. Confluent cultures of cells in 10-cm Petri dishes were washed three times with DMEM and incubated with 4 ml of serum-free DMEM for 24 hr. Eight milliliters of conditioned medium was collected and concentrated to 40 μl by Minicon (Amicon). Cells were scraped off the dishes with a rubber policeman and centrifuged at 150 × *g* for 5 min; the pellets were solubilized in 50 μl of lysis buffer [0.15 M NaCl/50 mM Tris·HCl, pH 7.4/1% Triton X-100/1% deoxycholate/0.1% sodium dodecyl sulfate (SDS)/10 mM EDTA/1 mM phenylmethylsulfonyl fluoride/150 kallikrein inhibitor units of aprotinin per ml] and clarified by centrifugation at 10,000 × *g* for 20 min. Samples were subjected to SDS gel electrophoresis in 10–18% polyacrylamide gradient gels under reducing conditions (11) and transferred to nitrocellulose membranes in a buffer containing 10% (vol/vol) ethanol, 150 mM glycine, and 20 mM Tris·HCl, pH 8.4, at 200 mA. The nitrocellulose membranes were incubated in 150 mM NaCl/10 mM Tris·HCl, pH 7.4/10% bovine serum albumin to block nonspecific binding, then incubated in a 1:50 dilution of a PD-ECGF-specific rabbit antiserum (5), and washed twice with 150 mM NaCl/10 mM Tris·HCl, pH 7.4, and twice with 150 mM NaCl/10 mM Tris·HCl, pH 7.4/0.05% Triton X-100. The nitrocellulose membranes were then incubated with ^{125}I-labeled staphylococcal protein A (5×10^5 cpm/ml) and washed as described above. Blots were subjected to autoradiography.

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Abbreviations: PD-ECGF, platelet-derived endothelial cell growth factor; FGF, fibroblast growth factor; FCS, fetal calf serum.

§To whom reprint requests should be addressed.

Northern Blot Analysis. Total RNA was extracted from cells by a lithium chloride/urea method described by Auffray and Rougeon (12). Cells were disintegrated in a buffer consisting of 3 M LiCl, 6 M urea, 0.2% SDS, and 0.1% Antifoam A (Sigma). After an incubation overnight on ice, the solution was centrifuged for 20 min at 16,000 × *g*. The resulting pellet was dissolved in a buffer consisting of 10 mM triethanolamine, pH 7.5, 1 mM EDTA, and 0.5% SDS and was extracted once with phenol followed by chloroform/isoamyl alcohol (24:1 vol/vol). The RNA was precipitated with 0.1 vol of 3 M sodium acetate and 2.2 vol of ethanol. All RNA samples were subjected to oligo(dT)-cellulose chromatography to select poly(A)$^+$ RNA before the electrophoresis.

RNA samples (10 μg per lane) were electrophoresed in a formaldehyde/agarose gel [0.8% agarose/2.2 M formaldehyde, 40 mM 3-(*N*-morpholino)propanesulfonic acid (Mops)/2 mM EDTA/10 mM sodium acetate, pH 7.0] and transferred by blotting to a nitrocellulose filter. The filters were prehybridized and hybridized in a buffer consisting of 20% (wt/wt) formamide, 5 × SSC (1× SSC consists of 0.15 M NaCl and 0.015 M trisodium citrate, pH 7.0), 5× Denhardt's solution (1× Denhardt's solution consists of 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone), 5 mM Na_2HPO_4, 5 mM NaH_2PO_4, 0.1% SDS, and salmon sperm DNA at 200 μg/ml. All hybridizations were carried out at 42°C. A PD-ECGF cDNA probe (pPL5; ref. 3) was ^{32}P-labeled with a multiprime labeling kit (RPN.1601, purchased from Amersham) to a specific activity of approximately 10^9 cpm/μg of DNA. After 4 hr preincubation the filters were hybridized with the radioactive cDNA probe for 12 hr. The filters were washed in 2× SSC/0.5% SDS for 30 min at 65°C, followed by a wash in 0.1× SSC/0.1% SDS at the same temperature. The filters were autoradiographed by using a Kodak XAR-5 film at −70°C in the presence of intensifying screens.

The amount of mRNA loaded in each lane was checked by ethidium bromide staining of the agarose gel and by hybridizing the filter to a glyceraldehyde-3-phosphate dehydrogenase cDNA probe (pHcGAP$_3$, obtained from Ray Wu, Cornell University, Ithaca, NY) (13).

Biosynthetic Labeling and Immunoprecipitation. Confluent cultures of cells in 60-mm dishes were washed three times with Dulbecco's phosphate-buffered saline (PBS) and given 1 ml of methionine-free and cysteine-free MCDB 104 medium (GIBCO) supplemented with 20 mM Hepes at pH 7.4, bovine serum albumin at 1 mg/ml, and 0.5 mM $CaCl_2$. [^{35}S]Methionine (0.25 mCi/ml; 1 Ci = 37 GBq) and [^{35}S]cysteine (0.25 mCi/ml) were then added and the cells were incubated at 37°C for various time periods. After incubation, the conditioned media were collected; the cells were washed with PBS and either lysed immediately or, for chase experiments, further incubated for various time periods in a medium containing 5-fold molar excesses of unlabeled methionine and cysteine. The cells were then solubilized in 1 ml of lysis buffer and clarified, as described above. The cell lysates and conditioned media were cleared three times with nonimmune serum and then divided into aliquots. Samples were incubated at 4°C for 2 hr with 2 μl of nonimmune serum or the specific rabbit antiserum to PD-ECGF and given 60 μl of protein A-Sepharose (Pharmacia) slurry (50% packed beads in 0.15 m NaCl/20 mM Tris·HCl, pH 7.4/0.2% Triton X-100). The mixture was incubated for another 45 min at 4°C with gentle mixing. The beads were then spun down and subsequently washed six times with lysis buffer, four times with 0.5 M NaCl/20 mM Tris·HCl, pH 7.4/0.2% Triton X-100, and finally once with only 20 mM Tris·HCl, pH 7.4. The material adsorbed to the beads was then eluted by addition of 40 μl of SDS sample buffer (11) and heating at 95°C for 3 min. Samples were analyzed by SDS gel electrophoresis using 10–18% polyacrylamide gradient gels under reducing conditions (11), followed by fluorography.

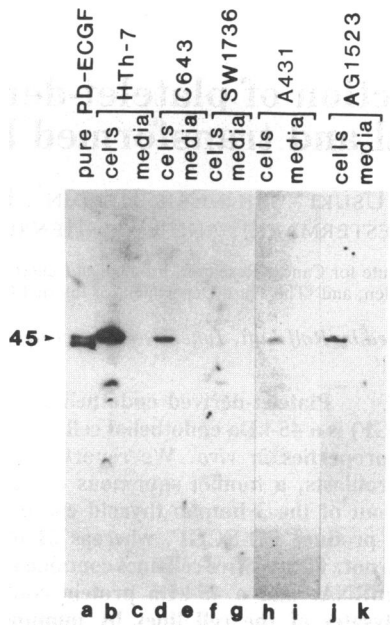

FIG. 1. PD-ECGF antiserum immunoblot analysis of cell lysates and conditioned media of human anaplastic thyroid carcinoma cell lines and human fibroblasts. Cell lysates (lanes b, d, f, h, and j) and conditioned media (lanes c, e, g, i, and k) of HTh-7 cells, C 643 cells, SW 1736 cells, A 431 cells, and human foreskin fibroblasts (AG 1523) were analyzed. PD-ECGF purified from platelets was run in parallel (lane a); it occurs as a doublet of about 45 kDa, probably due to proteolysis during preparation (5).

Assay of Endothelial Cell Growth Promoting Activity. Porcine aortic endothelial cells, cultured in Ham's F-10 medium supplemented with 10% FCS and antibiotics (2), were treated with trypsin and replated sparsely (≈1 × 10^4 cells per well) in 500 μl of Ham's F-10 medium containing 0.5% FCS in 24-well tissue culture plates. After 24 hr of incubation, samples were added to the wells. Eighteen hours later, [^{3}H]thymidine (0.2 μCi per well, 6.7 Ci/mol; New England Nuclear) was added. After an additional 6 hr, the cells were fixed with ice-cold 5% (wt/vol) trichloroacetic acid for 20

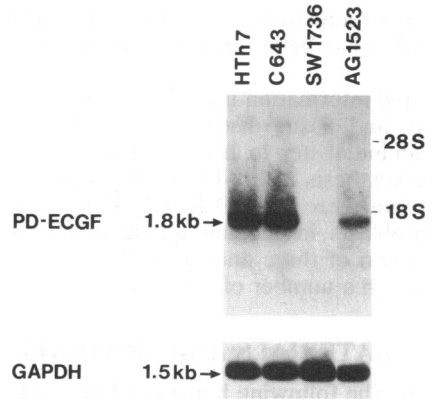

FIG. 2. Expression of PD-ECGF mRNA in human anaplastic carcinoma cell lines and normal fibroblasts. Poly(A)$^+$ RNA (10 μg per sample) from three different anaplastic thyroid carcinoma cell lines (HTh-7, C 643, and SW 1736) and normal foreskin fibroblasts (AG 1523) was electrophoresed, blotted, and hybridized. The filter was hybridized with a PD-ECGF cDNA probe and, as a control of the amount of mRNA loaded, with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. Positions of rRNAs are shown on the right.

min. The resulting precipitates were washed extensively with water and solubilized with 200 μl of 1 M NaOH. After incubation at room temperature for 20 min, 250 μl of 1 M HCl was added to the wells. ^{3}H radioactivity was then determined in a liquid scintillation counter, using 10 ml of Insta-Gel (Packard) per sample.

Lysates of cells for assay of growth-promoting activity were prepared as follows. Confluent cultures of cells in Petri dishes were washed three times with DMEM and then incubated in 4 ml of Ham's F-10 medium containing 0.5% FCS for 24 hr. Conditioned medium was collected and the cells were washed three times with PBS and scraped off the plate with a rubber policeman. After centrifugation at 150 × *g* for 5 min, the cell pellets were suspended in 50 μl of 50 mM Tris·HCl, pH 7.4/0.15 M NaCl and then lysed with five cycles of freezing and thawing. The lysate was clarified by centrifugation at 10,000 × *g* for 20 min.

RESULTS

PD-ECGF Is Produced by Human Foreskin Fibroblasts and Carcinoma Cell Lines. Cell lysates and conditioned media of a number of human cell lines of different histogenetic origins were subjected to immunoblotting using a specific rabbit antiserum to PD-ECGF (5). Analysis of the lysates of human foreskin fibroblasts, the anaplastic thyroid carcinoma cell lines HTh-7 and C 643, and the squamous cell carcinoma cell line A 431 revealed bands of the expected size, 45 kDa (Fig. 1). A very weak band of 45 kDa was found also in the conditioned medium of HTh-7 but not in the conditioned medium of C 643 or A 431. A third anaplastic thyroid carcinoma cell line tested, SW 1736, and 19 other human tumor cell lines (7 malignant gliomas, 3 leukemias, 1 osteosarcoma, 2 malignant histiocytomas, 1 melanoma, 1 hepatoma, 1 kidney carcinoma, 2 colon carcinomas, and 1 bladder carcinoma, specified in *Materials and Methods*), as well as normal porcine thyroid follicle cells and porcine aortic endothelial cells, were negative (data not shown).

PD-ECGF mRNA Expression. A PD-ECGF cDNA probe (3) was used to screen for PD-ECGF mRNA expression in various cell lines. Human foreskin fibroblasts and the thyroid carcinoma cell lines in HTh-7 and C 643 were found to express a transcript of 1.8 kilobases (kb) (Fig. 2), which is the expected size (3). However, the third thyroid carcinoma cell line, SW 1736, lacked PD-ECGF transcripts (Fig. 2), as did a number of other cell lines tested (not shown).

Pulse–Chase Analysis of PD-ECGF Biosynthesis. The experiments described above provide conclusive evidence that human foreskin fibroblasts (AG 1523), a squamous cell carcinoma cell line (A 431), and two anaplastic thyroid carci-

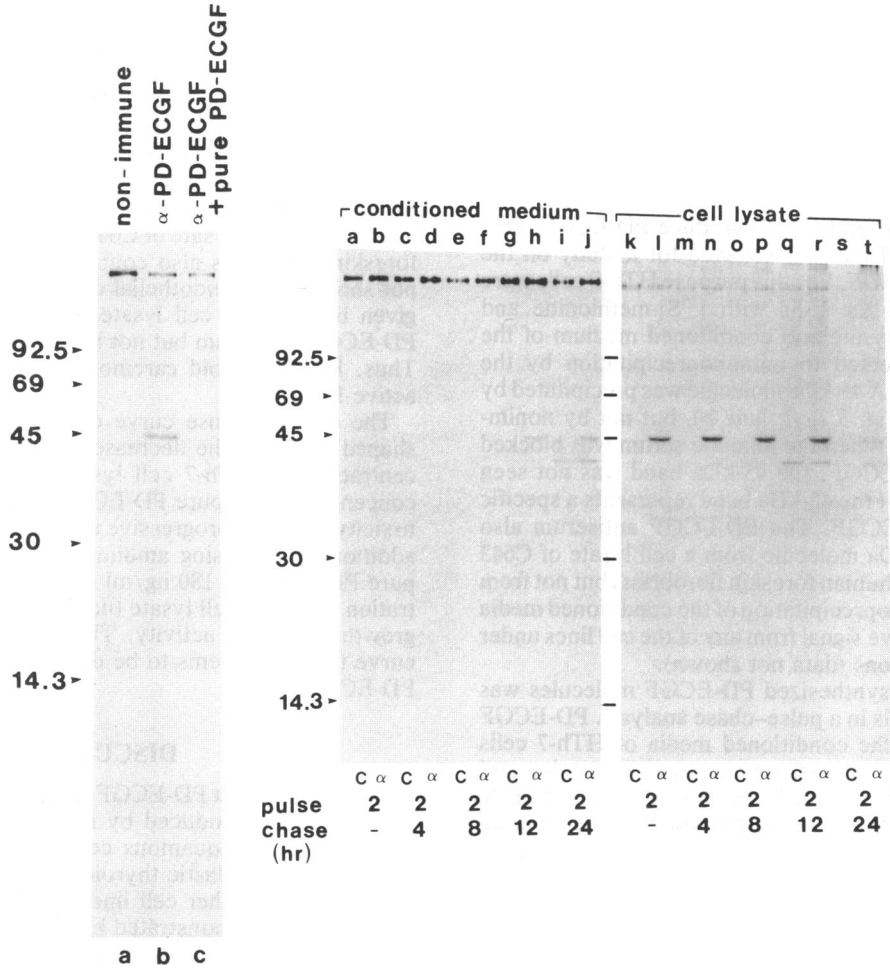

FIG. 3. Biosynthesis of PD-ECGF in HTh-7 cells. (*Left*) Immunoprecipitation of PD-ECGF from HTh-7 cells labeled in the presence of [^{35}S]methionine and [^{35}S]cysteine for 1 hr. A cell lysate was subjected to immunoprecipitation using a nonimmune serum (lane a) or a specific rabbit antiserum to PD-ECGF (lanes b and c) in the presence (lane c) or absence (lanes a and b) of 3 μg of PD-ECGF purified from human platelets. (*Right*) Pulse–chase analysis of PD-ECGF biosynthesis and processing in HTh-7 cells. Cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 2 hr and analyzed directly (lanes a, b, k, l) or after a chase with a 5-fold molar excess of unlabeled methionine and cysteine for 4 hr (lanes c, d, m, n), for 8 hr (lanes e, f, o, p), for 12 hr (lanes g, h, q, r), or for 24 hr (lanes i, j, s, t); cell lysates (lanes k–t) and conditioned media (lanes a–j) were immunoprecipitated with nonimmune serum (C; lanes a, c, e, g, i, k, m, o, q, s) or the specific PD-ECGF antiserum (α; lanes b, d, f, h, j, l, n, p, r, t). Positions of markers are indicated by their mass in kDa.

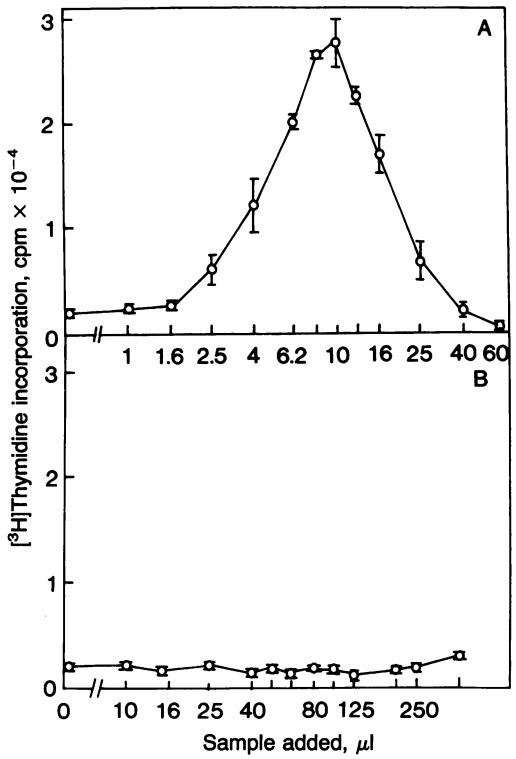

FIG. 4. Analysis of growth-promoting activity of cell lysate (*A*) and conditioned medium (*B*) of HTh-7 cells with porcine aortic endothelial cells as target cells. Values represent mean ± SEM of triplicate samples.

noma cell lines (HTh-7 and C 643) produce PD-ECGF. One of these cell lines, HTh-7, was selected for a study on the biosynthesis of PD-ECGF. For this purpose HTh-7 cells were metabolically labeled for 1 hr with [^{35}S]-methionine and [^{35}S]cysteine. A cell lysate and conditioned medium of the cells were then subjected to immunoprecipitation by the PD-ECGF antiserum. A 45-kDa molecule was precipitated by the immune serum (Fig. 3 *Left*, lane b), but not by nonimmune serum (lane a). When the immune serum was blocked by 3 μg of pure PD-ECGF, the 45-kDa band was not seen (lane c), indicating that the 45-kDa band represents a specific precipitation of PD-ECGF. The PD-ECGF antiserum also brought down a 45-kDa molecule from a cell lysate of C643 cells, A 431 cells, and human foreskin fibroblasts but not from SW 1736 cells; immunoprecipitation of the conditioned media did not give any positive signal from any of the cell lines under these labeling conditions (data not shown).

The maturation of synthesized PD-ECGF molecules was followed in HTh-7 cells in a pulse–chase analysis. PD-ECGF was not detected in the conditioned media of HTh-7 cells pulsed for 2 hr with [^{35}S]methionine and [^{35}S]cysteine and then chased for 6 hr (Fig. 3 *Right*, lanes b, d, f). The 45-kDa PD-ECGF molecule appeared in the conditioned medium from 12 hr of chase (lane h) and increased after 24 hr of chase (lane j), indicating that PD-ECGF was released from HTh-7 cells, but only very slowly. A 40-kDa protein was precipitated from the conditioned medium after 12 and 24 hr of chase (lanes h, j). This was considered nonspecific, since it was also precipitated by nonimmune serum (lanes g and i).

In the cell lysate, the 45-kDa molecule was seen after 2 hr of pulse (lane l) and was unchanged in size after chase for different times (lanes n, p, r). The nonspecific 40-kDa band was also seen in the cell lysate after 12 and 24 hr of chase (lanes q, r, s, t). Only after 24 hr of chase (lane t) did the 45-kDa PD-ECGF decrease considerably and a 35-kDa protein appeared, which may be a degradation product of PD-ECGF. No precursor forms of PD-ECGF were observed, even when a pulse as short as 15 min was used (data not shown).

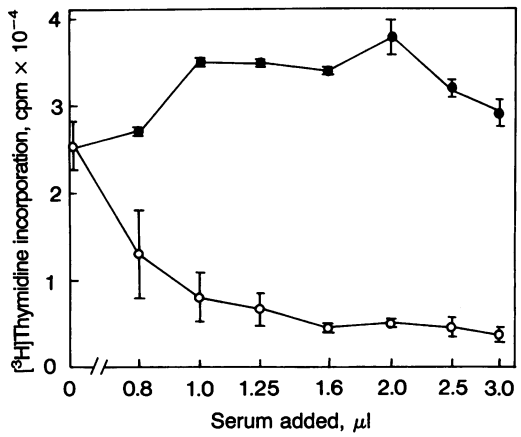

FIG. 5. Endothelial cell growth-promoting activity of HTh-7 cell lysate. Porcine aortic endothelial cells whose growth had been arrested with a low concentration of serum (0.5%) in the medium were incubated with 10 μl of HTh-7 cell lysate and the indicated volumes of immune serum (○) or nonimmune serum (●). Values are presented as means and range of duplicates.

Biological Activity of PD-ECGF Expressed in Thyroid Carcinoma Cells. The mitogenic activities of cell lysates and conditioned media of thyroid carcinoma cell lines were examined with porcine aortic endothelial cells used as target cells. Conditioned medium of HTh-7 cells had no significant growth-promoting activity on endothelial cells, but a strong mitogenic activity was found in a cell lysate of this cell line (Fig. 4). Maximal activity was observed after addition of 10 μl of lysate; at higher concentrations the mitogenic activity decreased. A cell lysate of C643 cells, A 431 cells, and human foreskin fibroblasts also contained mitogenic activity (data not shown). The endothelial cell growth-promoting activity given by 10 μl of cell lysate was completely inhibited by PD-ECGF antiserum but not by nonimmune serum (Fig. 5). Thus, HTh-7 thyroid carcinoma cells produce biologically active PD-ECGF.

The dose–response curve of HTh-7 cell lysate was bell shaped (Fig. 4). The decrease in activity seen at high concentrations of HTh-7 cell lysate is also observed at high concentrations of pure PD-ECGF (Fig. 6*A*). It is not due to toxicity, because progressive neutralization of PD-ECGF by addition of increasing amounts of PD-ECGF antiserum to pure PD-ECGF at 180 ng/ml (Fig. 6*B*) or to a high concentration of HTh-7 cell lysate (not shown) led to an increase in growth-promoting activity. The bell-shaped dose–response curve therefore seems to be due to an intrinsic property of PD-ECGF.

DISCUSSION

We report here that PD-ECGF, originally identified in human platelets (1), is produced by normal human foreskin fibroblasts, a human squamous cell carcinoma, and by two of three human anaplastic thyroid carcinoma cell lines but not by a variety of other cell lines. PD-ECGF production was unequivocally demonstrated by Northern blot hybridization analysis, immunoblotting, and the presence of a functionally active component, the activity of which could be blocked by PD-ECGF antibodies.

Anaplastic thyroid carcinoma is a highly malignant, rapidly growing neoplasm. The current view is that the progressive growth of a malignant neoplasm requires an efficient neovascularization, induced by tumor-derived angiogenic substances (14). Recent studies have shown that the expression of a transfected PD-ECGF cDNA in NIH 3T3 cells elicits a

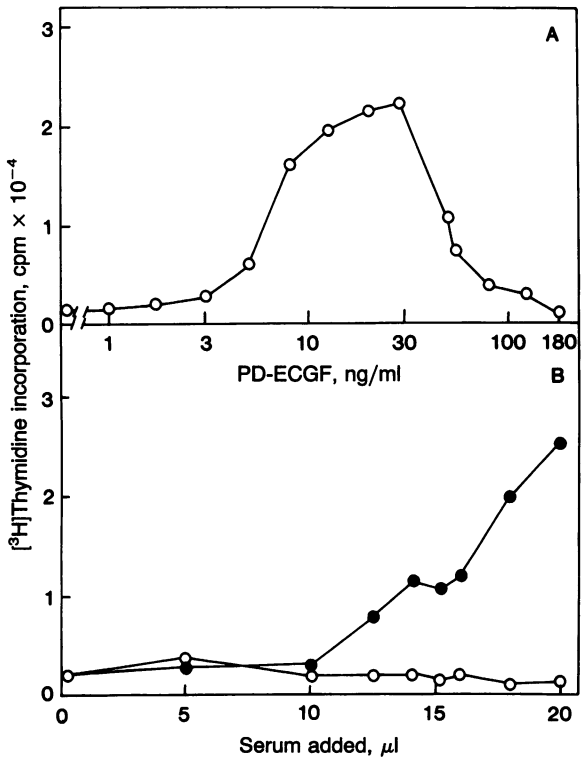

FIG. 6. (*A*) Dose–response curve of porcine aortic endothelial cells to pure PD-ECGF. (*B*) Growth-promoting activity of PD-ECGF at 180 ng/ml in the presence of increasing volumes of a rabbit PD-ECGF antiserum (●) or nonimmune serum (○). Values represent means of duplicates.

marked angiogenic response in tumors formed in *nude* mice (3). In view of this finding, it is interesting to note that anaplastic thyroid carcinoma cells express PD-ECGF mRNA and synthesize a biologically active product. Our findings thus imply that PD-ECGF may be a mediator of angiogenesis in thyroid carcinoma. We found no effect of PD-ECGF on the growth of normal porcine thyroid cells or of any of the thyroid carcinoma cell lines (data not shown). It is therefore unlikely that PD-ECGF acts as an autocrine growth factor for such cells.

It is noteworthy that PD-ECGF was also found to be produced by a normal foreskin fibroblast cell line. This suggests that a regulated expression of PD-ECGF may occur in nonmalignant conditions. Whether other normal cell types produce PD-ECGF remains to be investigated.

The mitogenic response of endothelial cells to PD-ECGF produced by HTh-7 cells was found to be bell shaped (Fig. 4). Similarly, the response to basic FGF has been found to decrease when it is present at high concentrations (15, 16). The mechanism behind this phenomenon remains to be determined; since the receptor for PD-ECGF and its post-receptor signaling system have not yet been identified, we are at present unable to determine at which level the response is down-regulated.

Studies on the biosynthesis of PD-ECGF by HTh-7 carcinoma cells (Fig. 3) and by normal human fibroblasts (data not shown) revealed that PD-ECGF is synthesized as a 45-kDa component which is released only slowly and without further noticeable processing. The observed size is similar to that of purified PD-ECGF (2) and of the component recognized by PD-ECGF antibodies by immunoblotting (Fig. 1). The open reading frame of the PD-ECGF mRNA implies the synthesis of a 50-kDa protein. This product is apparently processed to an 49-kDa species by loss of 10 N-terminal amino acids (3). It is possible that this processing occurs very fast, since it was not noticed in a pulse–chase analysis (Fig. 3). Whether some amino acids are cleaved from the C terminus also is not known. The slow release of PD-ECGF from HTh-7 cells is compatible with the lack of signal sequence in the primary translational product (3). It is completely unclear by which route the factor is externalized. An obvious possibility is that it is released from dying and disintegrating cells. Alternatively, the factor is released by a controlled mechanism, the nature of which remains to be elucidated. It is notable that the lack of signal sequence and slow release are features that PD-ECGF shares with two other angiogenic factors, acidic (6) and basic (7) FGF. With regard to the latter substances, it has been suggested that they are sequestered in an intracellular compartment to prevent them from eliciting an unscheduled mitogenic and angiogenic response in normal resting tissues (17).

Our present study shows that PD-ECGF is not a specific platelet product; its expression is, however, rather restricted. The recent finding (3) that PD-ECGF is a potent angiogenic factor makes studies on its occurrence in normal and neoplastic tissues important. Further and more extensive investigations on the expression of PD-ECGF in normal and malignant cells are therefore warranted.

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