The Role of Platelet-derived Growth Factor Production by Tumor-associated Macrophages in Tumor Stroma Formation in Lung Cancer

Jean-Michel Vignaud, Béatrice Marie, Nathalie Klein, François Plénat, Michael Pech, Jacques Borrelly, Nadine Martinet, Adrien Duprez, and Yves Martinet


ABSTRACT

Lung cancer is the most common cause of death by cancer in developed countries. Since a tumor cannot develop without the parallel expansion of a tumor stroma, a better understanding of its formation could lead to new therapeutic approaches. In this respect, since platelet-derived growth factor (PDGF) is a chemotactic and growth factor for mesenchymal and endothelial cells, lung tumors of patients undergoing surgery for non-small cell lung cancer were evaluated for their replication rate using iododeoxyuridine incorporation, and for the expression of PDGF genes and the presence of PDGF A and B chains and of PDGF receptor α and β subunits.

This observation demonstrates that: (a) tumor cells and stroma mesenchymal cells, but not tumor-associated macrophages, display a high replication rate; (b) 1 of 3 tumors are characterized by cancer cells expressing the genes for PDGF A and/or B chains, while 1 of 2 tumors are composed of tumor cells presenting PDGF receptors α and β subunits on their surface, and in only 1 of 6 tumors, tumor cells express PDGF and its receptor; (c) in almost all tumors, tumor-associated macrophages express PDGF A and/or B chain genes; (d) mesenchymal cells, as well as endothelial cells, do not express PDGF A and B chain genes but do express PDGF receptor α and β subunits; and (e) an ongoing active process was suggested in the periphery of the tumor by the simultaneous strong expression of PDGF A and B chain genes by tumor-associated macrophages and the high replication rate of mesenchymal and endothelial cells in the same area.

Thus, PDGF is likely to have a limited autocrine role in tumor cell replication but is a potential player, in a paracrine fashion, in tumor stroma development.

INTRODUCTION

Malignant tumor progression is characterized by the simultaneous proliferation of cancer cells and the development of tumor stroma (1). Tumor stroma is composed of different cell types including endothelial, mesenchymal, and inflammatory cells (lymphocytes, mononuclear phagocytes) (2). The presence of tumor stroma is a prerequisite for tumor growth, and the replication of both cancerous and stromal cells is currently thought to depend on the local presence of growth factors interacting with their specific receptors in autocrine and/or paracrine fashions (1, 3, 4).

PDGF3 is a potent chemotactic and growth factor for mesenchymal cells and endothelial cells. PDGF is composed of two chains, A and B, the B chain being coded for by the c-sis proto-oncogene (5–7). PDGF interacts on target cells with a receptor composed of two subunits, α and β. The α subunit binds PDGF A and B chains, while the β subunit only binds PDGF B chain with high affinity (8–11). Besides being involved, in a paracrine fashion, in different human fibrotic disorders [atherosclerosis, lung fibrosis (12–15)], PDGF has been suggested to participate, in an autocrine fashion, in the abnormal replication of some cancer cells (16).

Lung cancer is the most common cause of death by cancer in developed countries, due to its high incidence and its low 5-year survival rate (about 10%) (17). Since a tumor cannot grow without significant tumor stroma, a better understanding of tumor stroma formation could help in conceiving new therapeutic approaches (1). Thus, the potential involvement of PDGF in tumor stroma formation in lung cancer was evaluated.

Tumors obtained from patients undergoing surgery for nonsmall cell lung cancer were evaluated by immunohistochemistry and immunofluorescence using antibodies directed against PDGF A and B chains, and against PDGF receptor α and β subunits, and by in situ hybridization with the corresponding probes. Furthermore, the replicating status of the cells was evaluated by IdUrd incorporation.

MATERIALS AND METHODS

Study Populations

Patients with non-small cell lung cancer (n = 64: 60 males, 4 females; mean age, 61 ± 9 years) not subjected to preoperative radiotherapy and/or chemotherapy were enrolled in this study after informed consent. All of these patients had a prior history of smoking. Lung cancer histological types were defined according to the WHO criteria (18): 36 squamous cell lung carcinomas and 28 lung adenocarcinomas. Disease staging after surgery and pathology was defined according to the Fifth World Conference on Lung Cancer Recommendations for non-small cell lung cancer (19): stage I, n = 26; stage II, n = 13; stage III, n = 20; and stage IV, n = 5.

Normal lung specimens obtained from nonsmokers undergoing surgery for a nonneoplastic lesion of the lung were evaluated as controls (n = 8: 5 males, 3 females; mean age, 41 ± 6 years).

DNA Replication Evaluation by 5-Iododeoxyuridine Incorporation

IdUrd, a halogenated pyrimidine deoxyribonucleoside, is incorporated in vivo (in competition with thymidine) into newly synthesized DNA during the S phase of cells undergoing mitosis (20). The modified nucleotide incorporated into DNA is easily detected in nuclei on tissue sections by immunohistochemistry, using a specific monoclonal antibody (21). IdUrd (Fluka, Strasbourg, France) was prepared as a 1% (w/v) solution in a 5% (w/v) glucose solution, the pH was adjusted to 9.5 with a 1 M sodium hydroxyde solution, and the solution was filtered for sterilization. This solution was stable in the dark at 4°C for 10 days. The agreement to administer IdUrd to patients was delivered by the Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Nancy. After informed consent for each patient, IdUrd (1.4 g/m²) was administered (i.v.) over a period of 24 ± 1 h before surgery, as described before; no side effect was observed (22). The evaluation by immunohistochemistry of IdUrd incorporation is described in the next paragraph.

Immunohistochemistry

Tissue processing, histological staining, antibodies used, and the methods required for immunostaining were as follows.
Tissue Processing. Tumor specimens were collected between 20 and 60 min after the end of IdUrd administration. Several blocks were cut from each tumor. Some were snap-frozen in isopentane, precooled in liquid nitrogen, and stored in liquid nitrogen vapor until further processing; others were immersed into ethanol-acetic acid (3:1, v/v) for 12 h, and the last ones were fixed for 3 h in 4% paraformaldehyde in PBS, pH 7.4. Fixed samples were then routinely processed on automatic processor, and infiltrated with paraffin.

Control normal lung specimens were obtained: (a) when available from these patients, from a lobe free of any pathological evidence of malignancy; and (b) from control nonsmoker individuals undergoing lobectomy for a coin lesion of the lung.

Histological Staining. Sections of all paraffin-embedded blocks were dewaxed and brought to water through several washes with toluene and graded ethanol. They were then stained with hematoxylin and eosin.

Antibodies. For immunohistochemical studies we used monoclonal antibodies anti-PDGF A chain/pfa8, anti-PDGF B chain/vsb8a and anti-PDGF B chain/vsb9a, anti-bromodeoxyuridine/B-44 antibody (Becton Dickinson, San Jose, CA), PG-M1/CD68 antibody (Dako, Copenhagen, Denmark), anti-vimentin/LN6 antibody (Biogenex, San Ramon, CA), and anti-keratin/KL1 antibody (Immunotech, Marseille, France). Anti-PDGF A chain antibody/pfa8 is one of a panel of mouse monoclonal antibodies raised against human recombinant PDGF-AA and analyzed for their ability to inhibit the binding of human PDGF-AA to the PDGF receptor α subunit; it does recognize the PDGF-A chain specifically on Western blots. Anti-PDGF B chain/vsb8a and vsb9a monoclonal antibodies were raised against the purified v-sis gene product and recognize human PDGF-B but not PDGF-A chain (23). Anti-PDGF receptor α and β subunit antibodies (Genzyme, Boston, MA) were characterized previously not to bind to other known receptors (24—26) but to bind specifically to PDGF receptor α and β subunits. Anti-bromodeoxyuridine/B-44 antibody identifies cells with a dark brown granular staining, and with nuclear fast blue BB salt-0.1 M Tris-HCl (pH 8.2) produced a deep blue precipitate. The sections were then stained with BrdUrd/B-44 and/or anti-vimentin/LN6, anti-human von Willebrand factor antibody first, and the anti-BrdU/B44 antibody second, we identified proliferating endothelial cells, whereas anti-keratin/KL1 antibody recognizes epithelial cells.

Immunoenzymatic Staining. A preliminary assay displayed that anti-PDGF A chain/pfa8, anti-PDGF B chain/vsb8a and /vsb9a, PG-M1/CD68, anti-BrdUrd/B-44, anti-vimentin/LN6, anti-keratin/KL1, and anti-von Willebrand antibodies presented the same immunoreactivity on frozen and paraffin-embedded sections. Because a better morphology was obtained with the latter processing, samples evaluated with these antibodies were prepared from paraffin-embedded tissues. For this purpose, 5-μm sections from tissues fixed in ethanol-acetic acid were dewaxed through several baths of toluene and brought to PBS containing 0.1% BSA through graded ethanol. Expression of PDGF receptors was checked on 5-μm frozen sections fixed for 10 min in cold acetone (4°C), and then rinsed in PBS.

Sections stained with PG-M1/CD68, anti-human von Willebrand factor, and anti-vimentin/LN6 antibodies were preincubated for 10 min at 37°C in a solution of 0.1% trypsin (w/v)-0.4% calcium chloride (w/v) in Tris-HCl buffer (pH 7.6), rinsed in PBS for 10 min, and except for sections stained with PG-M1/CD68 antibody, all sections were processed in a microwave. For this purpose, the slides were dipped in a plastic jar filled with 10 mm citrate buffer (pH 6.0), processed 3 times (5 min each) at 750 W, cooled for 15 min to room temperature, and then rinsed in PBS for 5 min (28).

IdUrd incorporated into DNA was accessible to the anti-BrdUrd/B-44 antibody only after denaturation of cellular DNA. For this purpose, the corresponding sections were submitted to a chlorhydric hydrolysis by immersion for 15 min in 4 NHCl at room temperature, and then washed for 10 min under running tap water, with a final 5-mm wash in PBS. The sections were then incubated for 20 min in a nonfat dried milk solution to prevent background staining [30% (w/v) nonfat dried milk-3% (w/v) BSA-0.3% (v/v) Tween 20-0.1% (w/v) NaNO2 in distilled water].

Pretreated and nontreated sections were overlaid overnight at 4°C, with the appropriate monoclonal antibodies diluted (in PBS containing 0.1% BSA) to the following concentrations: anti-PDGF A chain/pfa8, 1:250; v/v; anti-PDGF B chain/vsb8a and /vsb9a, both 1:200, v/v; anti-BrdUrd/B-44, 1:150, v/v; PG-M1/CD68, 1:150, v/v; anti-PDGF receptor α and β subunits, 1:30 and 1:200, v/v, respectively, anti-vimentin/LN6, 1:80, v/v; anti-human von Willebrand factor, 1:150, v/v; and anti-keratin/KL1, 1:150, v/v. The sections were then washed in PBS, with two changes of 5 min each, and endogenous peroxidase activities were blocked by incubation for 30 min in a solution of 0.3% (v/v) H2O2-0.1% (w/v) NaNO2 in PBS. The samples were then rinsed in PBS (two changes of 5 min each) and incubated for 30 min with a peroxidase-conjugated rabbit anti-mouse antibody (Dako 1:30, v/v, in PBS containing 0.1% BSA). After further washing with PBS, the samples were incubated for 30 min with a peroxidase swine anti-rabbit antibody (Dako 1:30, v/v, in PBS containing 0.1% BSA). This step was followed by two washes in PBS before a final 5-min incubation in a Tris-HCl buffer (0.05 M, pH 7.6) containing diaminobenzidine (0.6 mg/ml and H2O2 (0.01%, v/v). After several washes with distilled water, the sections were mounted for microscopic examination after hematoxylin and eosin counterstaining. Positive cells were defined as those cells with a dark brown granular staining, and with nuclear localization for anti-BrdUrd/B-44 antibody and cytoplasmic localization for the other antibodies.

Immunofluorescence Study. Immunofluorescent microscopic technique was performed on 6-μm-thick frozen sections, fixed for 10 min in cold (4°C) acetone, rinsed for 5 min in PBS (0.1% BSA), and overlaid overnight at 4°C with the PDGF receptor α and β subunit antibodies (1:30, v/v, and 1:200, v/v, respectively) in PBS containing 0.1% BSA. After two washes in PBS for 5 min each, the sections were incubated for 30 min with a rabbit anti-mouse antibody (Dako) (1:30, v/v, in PBS-0.1% BSA), rinsed again in PBS, and incubated with a swine anti-rabbit fluorescein-conjugated antibody (Dako) (1:80, v/v, in PBS-0.1% BSA). After a final wash in PBS, the sections were mounted with an antifade mounting media (glycerin jelly containing 0.1 M p-phenylene diamine) and examined using a mercury lamp and the appropriate filter.

Double Staining Procedures. To fully confirm that PDGF-positive stroma cells were mononuclear phagocytes, a double immunofluorescence staining was performed on the same sections by sequential application of two different immunofluorescent sandwiches. Anti-PDGF A chain/pfa8 or B chain/vsb8a and /vsb9a antibodies were first applied on samples and visualized using a rabbit anti-mouse antibody (Dako) (1:30, v/v, in PBS-0.1% BSA), followed by a swine anti-rabbit fluorescein antibody (Dako) (1:80, v/v, in PBS-0.1% BSA). The PDGF A chain/pfa8 antibody was then overlaid on sections and visualized in the same way except that the goat anti-rabbit antibody was a tetramethyl rhodamine isothiocyanate conjugate (1:100, v/v; The Jackson Laboratory, West Grove, PA). The sections were examined using a mercury lamp and the appropriate filters.

A similar sequential immunoenzymatic method was used to evaluate the in situ replication of mononuclear phagocytes. The samples were first incubated with PG-M1/CD68 antibody and processed, as above, with two peroxidase-labeled secondary antibodies using diaminobenzidine as chromogen (providing a brown dark precipitate). The samples were then stained with BrdUrd/B-44 antibody, as described above, but visualized with two calf AP-conjugated secondary antibodies [AP rabbit anti-mouse antibody (1:30, v/v, in PBS-0.1% BSA); (Dako) and AP swine anti-rabbit fluorescein antibody (Dako) (1:80, v/v, in PBS-0.1% BSA)]. The PDGF A chain/pfa8 antibody was then overlaid on sections and visualized in the same way except that the goat anti-rabbit antibody was a tetramethyl rhodamine isothiocyanate conjugate (1:100, v/v; The Jackson Laboratory, West Grove, PA). The sections were examined using a mercury lamp and the appropriate filters.

The same sequential procedure was used to detect the expression of PDGF receptor α and/or β subunits on the surface of TAM (identified with PG-M1/CD68 antibody). Furthermore, using anti-PDGF A chain/pfa8 and B chain/vsb8a and vsb9a and anti-BrdUrd/B-44 antibodies, successively overlaid on sections, we evaluated the colocalization of macrophages producing PDGF and replicating mesenchymal and endothelial cells.

Controls. To test the specificity of the labeling protocols, the following controls were carried out on selected sections for each antibody: (a) omission of the incubation step with the primary antibody; (b) substitution of a nonimmune serum in place of the primary antibody; (c) omission of the incubation step with both primary and secondary antibodies; and for anti-BrdUrd/B-44 antibody, (d) processing of tissue sections from patients not infused with IdUrd before surgery. Furthermore, several controls of anti-PDGF A chain/pfa8 and B chain/vsb8a and vsb9a antibodies were carried out: (a) tissue sections (normal lung and lung cancer) were evaluated with these antibodies before or after incubation (90 min) of these antibodies with pure human platelet-purified PDGF.
PDGF homodimers and heterodimers, as well as with other relevant growth factors, such as insulin-like growth factor-1, fibroblast growth factors, tumor necrosis factor α, and TGF-β; and (b) anti-PDGF antibodies were tested for their ability to interact, in relevant bioassays, on the biological activities of other platelet-purified growth factors (TGF-α and TGF-β). These controls demonstrated that only PDGF chemotactic and “competence” growth activities were suppressed by incubation with these antibodies (not shown).

**Cell Quantification.** Cell numbers were evaluated by quantitative analysis with an optic grid (Zeiss Kpl-W) on nonconsecutive random fields. The IdUrd-labeling index was defined for tumor cells as the percentage of carcinoma cells with IdUrd-labeled nuclei among the total number of cancer cell nuclei scored (excluding stroma cells). For each tumor an average of 2500 cells was counted, dispatched in six microscopic fields, and randomly distributed, but three fields intersected the central part of the tumor, and three fields intersected the peripheral part. The same procedure was used to establish the IdUrd-labeling index of stroma cells (tumor-associated macrophages, fibroblasts, and endothelial cells). All cells were defined by classic histological criteria (the quality of the sections always allowed an easy and reliable identification of mononuclear macrophages and of the other cell types of the stroma), and further identified on adjacent sections by labeling with specific antibodies.

Mononuclear phagocytes producing PDGF were evaluated on adjacent sections, one with anti-PDGF A chain/pFA8 or B chain/vsb8a and vsb9a antibodies and the other with PG-M1/CD68 antibodies specifically recognizing monocytes-macrophages. For each biopsy, the number of PDGF-positive mononuclear phagocytes was evaluated and expressed as a percentage of the total number of macrophages present in the fields.

**In Situ Hybridization**

Sections (5 μm thick) were prepared from tissue samples fixed in paraformaldehyde, deparaffinized through several washes with toluene, and brought to PBS through graded ethanolns. The sections were then digested with pronase [0.12 mg/ml in 50 mM Tris-HCl (pH 7.4) 5 mM EDTA] for 10 min at room temperature, and incubated for 10 min in glycine (0.1 M in 0.1 M Tris-HCl, pH 7.4). The sections were then acetylated in anhydride acetic acid (0.5% in 0.1 M triethanolamine, pH 8.0) for 10 min, washed in PBS, and finally prehybridized at 52°C for 10 min in a solution of 50% formamide-2× SSC-100 μM dithiothreitol. Hybridization was performed overnight at 48°C in the following solution: 50% denatured formamide; 2× SSC (300 mM NaCl, 30 mM Na-citrate, pH 7.0), 100 mM dithiothreitol; 1 mg/ml yeast tRNA; 1 mg/ml sonicated salmon sperm DNA, 2 mg/ml BSA, and the 35S-labeled sense or antisense PDGF A-chain or B-chain RNA probes (0.6 × 10⁶ cpm/10 μl of hybridization solution).

Human PDGF B chain riboprobe was derived from the PDGF B chain template described previously (29). The PDGF A chain template was constructed by inserting a 681-base pair SacI-HindIII fragment of clone D1 (30) into pGEM-2 (Promega Corp., Madison, WI). The riboprobes were prepared according to the transcription protocol of Melton et al. (31) using 35S-labeled UTP nucleotide (Amersham, Les Ulis, France). The resulting transcripts were partially hydrolyzed with alkali so that most of the labeled fragments were 100–200 nucleotides in size (32).

Following hybridization, the sections were rinsed with formamide (50%, 2× SSC) at 50°C for 2 h, and digested with RNaseA for 30 min at 37°C according to Cox et al. (RNase A, 10 μg/ml; RNase T1, 500 units/ml; in 2× SSC) (32). The sections were washed again in formamide (50%, 2× SSC) for 2 h at 30°C, with a final wash in 1× SSC for 30 min at room temperature. Hybridized slides were autoradiographed with NTB2 emulsion (Kodak, Rochester, NY) and exposed at 4°C. Triplicate sections from each specimen were developed at weekly intervals over 3 weeks with Di9 Kodak developer (RNase A, 10 μg/ml; RNase T1, 500 units/ml; in 2× SSC) and hybridization with sense probes.

**RESULTS**

**Lung Cancer Cell Replication Status.** The tumor cells and different stroma cell types (mesenchymal cells, endothelial cells, and tumor-associated macrophages) were evaluated for their replication status with the use of the in vivo incorporation of IdUrd as a marker of DNA synthesis. The incorporated modified nucleotide was detected in nuclei with a monoclonal anti-BrdUrd antibody, and the different types of replicating cells were identified with the use of histological criteria and also with a double immunohistochemical procedure. In this respect, tissue sections were stained first with anti-keratin, anti-vimentin, anti-von Willebrand, or PG-M1/CD68 antibodies, binding to epithelial cells, mesenchymal cells, endothelial cells, and mononuclear phagocytes, respectively, and second with the anti-BrdUrd antibody. In all cases, and independently of the histological type of the tumor, carcinoma cells were characterized by a high labeling index with the anti-BrdUrd antibody, suggesting a high replication rate of these cells (Fig. 1a). In squamous cell carcinomas, the labeled nuclei were heterogeneously distributed across the tumor, with a high labeling index in the periphery of the tumors (40 ± 10% of replicating cells) in comparison to their central parts (26 ± 9% of replicating cells; P < 0.05 versus periphery). Furthermore, in well differentiated squamous cell carcinomas, the nuclei labeling of the tumor sheaths was restricted to the external tumor cell layers, while no staining was observed for the more central keratinized cells. In contrast, in lung adenocarcinoma, the labeled nuclei were homogeneously distributed across the tumors, with a mean of 28 ± 12% replicating cells.

Looking at the replication of mesenchymal and endothelial cells, we observed a clear difference between the status of the cells in the periphery of the tumor (making a ring in close contact with the normal surrounding lung tissue) and the status of the other cells present in the tumor. Indeed, in the central part of the tumor, fibroblasts and endothelial cells were scarcely replicating (<1 ± 0.5% replicating cells); however, in the peripheral part of the tumor closely connected with healthy alveolar spaces, 10 ± 4% of fibroblasts (Fig. 1b; P < 0.05 versus central part of the tumor) and 5 ± 2% of endothelial cells (Fig. 1c; P < 0.05 versus central part of the tumor) were replicating, suggesting an active proliferation of the stroma in the periphery of the tumor. This observation was identical for both histological types of non-small cell lung cancer. Finally, TAM-labeling index was always low with 1 ± 0.5% replicating macrophages (Fig. 1d), without any specific distribution among the tumor, and the same low replication rate was observed in squamous cell lung carcinoma and lung adenocarcinoma.

**PDGF and Cancer Cells.** The expression of PDGF A and B chain genes was evaluated by in situ hybridization with 35S-labeled antisense PDGF A-chain and PDGF B-chain riboprobes, and the presence of PDGF and PDGF receptor α and β subunit proteins was detected by immunohistochemistry using a panel of monoclonal antibodies (described in "Materials and Methods") directed against PDGF AA and BB homodimers and PDGF α and β receptor subunits. The expression of PDGF A (Fig. 2, a and b) and/or B (Fig. 2c) gene expression was observed in 19 tumors (30%), 10 squamous cell carcinomas, and 9 adenocarcinomas. mRNA expression was uniform among epithelial cells across the sections for each tumor, while PDGF protein detection (Fig. 2, d, e, and f) was characterized by a diffuse and strong cytoplasmic staining in 4 tumors and a patchy labeling (with 10–40% of the malignant cells stained) for the other 15 tumors, which was restricted to the external tumor cell layers, while no staining was observed for the more central keratinized cells. In contrast, in lung adenocarcinoma, the labeled nuclei were homogeneously distributed across the tumors, with a mean of 28 ± 12% replicating cells. Looking at the replication of mesenchymal and endothelial cells, we observed a clear difference between the status of the cells in the periphery of the tumor (making a ring in close contact with the normal surrounding lung tissue) and the status of the other cells present in the tumor. Indeed, in the central part of the tumor, fibroblasts and endothelial cells were scarcely replicating (<1 ± 0.5% replicating cells); however, in the peripheral part of the tumor closely connected with healthy alveolar spaces, 10 ± 4% of fibroblasts (Fig. 1b; P < 0.05 versus central part of the tumor) and 5 ± 2% of endothelial cells (Fig. 1c; P < 0.05 versus central part of the tumor) were replicating, suggesting an active proliferation of the stroma in the periphery of the tumor. This observation was identical for both histological types of non-small cell lung cancer. Finally, TAM-labeling index was always low with 1 ± 0.5% replicating macrophages (Fig. 1d), without any specific distribution among the tumor, and the same low replication rate was observed in squamous cell lung carcinoma and lung adenocarcinoma.

**Statistical Analysis**

All values are presented as mean ± SEM. All statistical comparisons were made using two-tailed Student's t-test.
PDGF PRODUCTION BY MACROPHAGES AND STROMA FORMATION

Fig. 1. Cell replication in human non-small cell lung cancer. 5-Iododeoxyuridine was infused the day before surgery to patients with lung cancer, and detected as described in “Materials and Methods” by immunohistochemistry with an anti-bromodeoxyuridine/B44 antibody on tissue sections of surgically resected tumors. Shown is one representative example obtained from a patient with squamous cell lung carcinoma. Replicating cells present dark brown nuclei (×1160). (a) Tumor cells with a high labeling index, especially in external layers of tumor sheets. (b) Frequently labeled mesenchymal cells in tumor stroma. (c) Capillary with numerous labeled endothelial cells. (d) Double immunolabeling showing that among tumor-associated macrophages stained in blue with PG-M1/CD68 antibody, an antibody specifically binding mononuclear phagocytes, only a few are replicating (brown nuclei, arrow).

Fig. 2. Malignant cell expression of PDGF A and B chains and of PDGF receptor α and β subunits in human non-small cell lung cancer. Surgically resected lung tumors were evaluated as described in “Materials and Methods” by in situ hybridization, immunohistochemistry, and immunofluorescence. Shown is one representative example obtained from a patient with squamous cell lung carcinoma (×1160). (a and c) In situ hybridization with PDGF A and B chain antisense probes, respectively (dark field examination). (b) Control of in situ hybridization with PDGF A sense probe. (d and f) Immunohistochemistry with anti-PDGF A/pfα8 chain (diffuse labeling) and B/pβα8 and /pβα9 chain (patchy staining) antibodies, respectively. (e) Control of immunohistochemistry with anti-PDGF A/pfα8 chain antibody previously incubated with platelet-purified PDGF. (g and i) Immunofluorescence with anti-PDGF receptor α (strong staining of both tumor and stroma cells) and β (the stroma is not stained in this strongly collagenized area, devoided of stromal cells) subunit antibodies, respectively. (h) Control of immunohistochemistry in the absence of incubation with either anti-PDGF receptor α subunit or β subunit antibody.
suggesting a heterogeneous synthesis or excretion of PDGF proteins. The specificity of the in situ hybridization and of the immunostaining reaction signals was controlled as described in "Materials and Methods." All controls produced negative results.

The presence of PDGF receptor α and β subunits on tumor cells was investigated by indirect immunofluorescence using two monoclonal antibodies specifically binding α and β PDGF receptor subunits (Fig. 2, g, h and i). Interestingly, PDGF receptor subunits on cancer cells were observed in 35 of 64 tumors (54%), and frequently in a dissociated fashion, with tumor cells presenting the α subunit alone in 17 of 35 tumors (26%), the β subunit alone in 4 of 35 specimens (6%), and both in 14 of 35 samples (22%). No difference was observed between squamous cell carcinoma and adenocarcinoma in respect to PDGF receptor α and/ or β subunit expression by cancer cells. Interestingly, only 11 of the 19 tumors expressing PDGF genes and producing PDGF protein were found to present PDGF receptor α and/or β subunits on the surface, suggesting a possible autocrine loop involving PDGF. Finally, all epithelial cells of the control lung samples neither expressed PDGF A and B chain genes nor were labeled with the antibodies directed against PDGF A and B chains and PDGF receptor α and β subunits (not shown).

PDGF and Tumor-associated Macrophages. The same tumor sections were evaluated for TAM status, demonstrating that in 59 of 64 lung cancers (92%), the macrophages present in tumor stroma expressed PDGF A and/or B chain genes (Fig. 3, a and b) and produced PDGF proteins (Fig. 3, c and d). A double indirect immunofluorescence labeling with anti-PDGF A/pfa8a chain and B/vsb8a chain antibodies, and the PG-M1/CD68 antibody, specifically binding mononuclear phagocytes, fully attested the monocyte-macrophage nature of the stroma cells labeled with these anti-PDGF antibodies (Fig. 3, e and f). These PDGF-producing TAM were mainly found in the peripheral part of the tumor, close connection with the normal surrounding lung tissue. Quantification was established in this site on serial sections. TAM-producing PDGF were quantified versus all mononuclear phagocytes identified with the PG-M1/CD68 antibody. A few tumors (n = 9, 14%) contained less than 5% of PDGF-positive TAM. But the majority of specimens (n = 50, 78%) contained 10–25% of PDGF-positive TAM. A gradient of PDGF-producing TAM was observed, with a maximum in the peripheral part of the tumor, and a progressive decrease toward the central part of the tumor. On the other hand, only a few PDGF-positive TAM were detected in the normal alveolar walls in close contact with the tumor. Furthermore, local accumulation of PDGF-producing TAM was observed within two normal structures included in the tumor, bronchi and some small intrapulmonary lymph nodes. Among PDGF-positive TAM, some produced PDGF A chain and the others PDGF B chain (Fig. 3g), but usually more PDGF A-positive than PDGF B-positive cells were counted.

TAM were evaluated for the presence of PDGF receptor α and/or β subunits using a double immunolabeling procedure (anti-PDGF receptor α and/or β subunit antibodies and PG-M1/CD68 antibody were sequentially applied). With either immunofluorescence or immunohistochemistry, a clear and certain observation could not be done. Indeed, TAM were located within a close meshed network of fibroblast processes strongly expressing PDGF receptor α and/or β subunits (not shown). Thus, it was often impossible to determine whether the cytoplasmic membrane staining was relevant to TAM and/or fibroblasts. However, in a few areas devoid of fibroblasts, some TAM were clearly stained with PDGF receptor α and/or β subunit antibodies. In this respect, it was not possible to extend these observations to other areas. Furthermore, endothelial macrophages present in the healthy tissue in close contact with tumor did not express PDGF α and/or β receptor subunits. Interestingly, a simultaneous evaluation of IdUrd incorporation and of PDGF production by a double immunohistochemistry procedure showed that both replicating mesenchymal cells and PDGF-positive TAM were observed in the same areas (Fig. 3h), suggesting a possible direct relationship. Finally, concerning PDGF-positive TAM, no difference was noticed between lung adenocarcinoma and squamous cell carcinoma.

PDGF and Tumor Stroma Mesenchymal Cells. Tumor stroma cells, such as fibroblasts and arterial smooth muscle cells, did not express either PDGF A or B chains at the gene and protein levels. In a similar fashion, connective tissue cells of the control lung specimens also failed to demonstrate any staining for these proteins and the corresponding genes. Sections were processed for receptor subunits by indirect immunofluorescence and immunoenzymatic methods with the monoclonal antibodies specifically binding α and β receptor subunits. PDGF receptor α and β subunits were strongly present in all tumors on mesenchymal cells (Fig. 4, a, b and c); this observation is consistent with the presence of both α and β subunits on the connective tissue cells of control lung sections (not shown). The great majority of cancer stroma mesenchymal cells coexpressed PDGF receptor α and β subunits (n = 54, 85%), but some expressed them in a dissociated fashion [α alone (n = 2, 3%) or β alone (n = 8, 12%)]. The nature of mesenchymal cells was further identified on immunohistochemical criteria (labeling with anti-vimentin/LN6 antibody).

PDGF and Endothelial Cells. Similar to mesenchymal cells, tumor-associated endothelial cells did not express PDGF A or B chains at the gene or protein levels, but did express PDGF receptor α and β subunits in all tumors and, more specifically, on small vessels (Fig. 4d).

DISCUSSION

This observation demonstrates that in non-small cell lung cancer: (a) tumor cells and stroma mesenchymal cells, but not TAM, display a high replication rate; (b) 1 of 3 tumors are characterized by cancer cells expressing the genes for PDGF A and/or B chains, while 1 of 2 of tumors are composed of tumor cells presenting PDGF receptors α and β subunits on their surface, and in only 1 of 6 tumors, tumor cells coexpress PDGF and its receptor; (c) in almost all tumors TAM express PDGF A and/or B chain genes; (d) mesenchymal cells, as well as endothelial cells, do not express PDGF A and B chain genes, but do express PDGF receptor α and β subunits; and (e) an ongoing active process was suggested in the periphery of the tumor by the simultaneous strong expression of PDGF A and B chain genes by TAM and the high replication rate of mesenchymal cells in the same area.

Due to its high incidence and to the poor efficiency of therapy, lung cancer is the most common cause of death by cancer in developed countries (17, 33). A better understanding of the relationships between the tumor and its host could help in designing new therapeutic approaches using, for example, inflammatory cells (lymphocytes, monocytes). If an abnormal cancer cell replication rate results from sequential genetic events allowing an unregulated proliferation of clones, however, a tumor cannot grow without the parallel installation of a tumor stroma made of noncancerous components (vessels, inflammatory cells, mesenchymal cells, and extracellular matrices) (1, 2, 34, 35). The stroma is the skeleton, the feeding support of the tumor, and the site of immunological relationships between cancer cells and the host. If, initially, tumor stroma development is directly under the control of tumor cells, later, its regulation is the result of complex and intricate relationships between cancer cells and stroma cells, specifically mesenchymal cells and inflammatory cells (34). Although the pronostic value of tumor stroma components in lung cancer is not known, their importance and type have been suggested to be correlated with the prognosis (1). The potential role of tumor-
Fig. 3. TAM expression of PDGF A and B chains in human non-small cell lung cancer. Surgically resected lung tumors were evaluated as described in “Materials and Methods” by in situ hybridization, immunohistochemistry, and immunofluorescence. Shown is one representative example obtained from a patient with a lung adenocarcinoma (×1160; except for a, ×2900). (a and b) In situ hybridization with PDGF A and B chain antisense probes, respectively (dark field examination). (c and d) Immunohistochemistry with anti-PDGF-A/p40 and B/va9a and /vsb9a chain antibodies, respectively, showing numerous PDGF-positive tumor-associated macrophages in the stroma. (e and f) Double immunofluorescence labeling with PG-M1/CD68 antibody (rhodamine labeling, red fluorescence) and anti-PDGF A/p40 chain antibody (fluorescein labeling, green fluorescence), respectively, demonstrating that PDGF-positive stromal cells are mononuclear phagocytes. (g) Double immunolabeling with anti-PDGF A/p40 chain antibody (blue staining) and anti-PDGF B/va9a and /vsb9a chain antibodies (brown staining), showing that tumor-associated macrophages can express PDGF AA and BB dimers. (h) Double immunostaining demonstrating the colocalization of TAM-producing PDGF B dimers (brown labeling) and replicating mesenchymal cells (blue anti-BrdUrd/B-44 antibody-labeled elongated nuclei).

Fig. 4. Tumor stroma cell expression of PDGF receptor α and β subunits in human non-small cell lung cancer. Surgically resected lung tumors were evaluated as described in “Materials and Methods” by immunohistochemistry and immunofluorescence. Shown is one representative example obtained from a patient with squamous cell lung carcinoma. (a and b) Immunofluorescence staining of mesenchymal cells with anti-PDGF receptor α and β subunit antibodies, respectively. Tumor cells are unlabeled (×750). (c) Delicate staining of the cytoplasmic membrane of fibroblasts with an anti-PDGF receptor β subunit antibody (×2900). (d) Endothelial cells from arteriole and capillaries exhibiting a clear immunofluorescence labeling with the anti-PDGF receptor α subunit antibody (×750).
associated inflammatory cells is not yet fully understood, since TAM have been shown to be able to exert a cytotoxic activity against tumor cells, but also, in some conditions, to help tumor cell replication (37, 38). In this respect, a better understanding of the origin and the functions of the different cell types involved in tumor stroma formation should help in the conceptual approach to using inflammatory cells as a therapeutic tool.

In order to better understand the mechanisms leading to tumor stroma formation, PDGF was evaluated, since this cytokine has been shown to participate in the pathogenesis of fibrosis in different organs including the lung (12–15). PDGF is a 30,000 cytokine initially purified from platelets, and produced by different types of normal cells (blood monocytes, alveolar macrophages, vascular smooth muscle cells, endothelial cells) and by some cancer cells. PDGF is composed of two chains, A (M, 14,000) and B (M, 17,000), linked by disulfide bonds and respectively coded for by a gene on chromosome 7 and the c-sis proto-oncogene on chromosome 22 (5–7, 39–42). PDGF molecules are active when composed of two chains (AA, AB, or BB). The PDGF receptor is composed of two subunits (α and β) and can be present under 3 different forms, αα, αβ, or ββ (8–11). The α subunit binds PDGF A and B chains, while the β subunit only binds PDGF B chain with high affinity. PDGF has been strongly suggested to play a role in wound healing and several diseases characterized by the local accumulation of mesenchymal cells, such as atherosclerosis and lung fibrosis (12–15). Interestingly, in both these diseases, mononuclear phagocytes have been suggested to play a direct role by expressing PDGF A and B chain genes and by releasing PDGF (12, 13, 43–45).

PDGF involvement in cancer is complex. In 1981, the sequence of the viral oncogene (v-sis) of the simian sarcoma virus was shown to share close sequence similarities with PDGF B chain gene (46, 47). It was then shown that the abnormal production of PDGF by a cell transfected by this virus could stimulate this cell replication in an autocrine fashion, resulting in cell transformation (48, 49). In this respect, only cells expressing PDGF receptor subunits could be transformed by this abnormally high production of PDGF (16). PDGF is frequently produced by different tumor cell types. There is no direct evidence that this high expression results from a specific mutation of the gene coding for PDGF, but may result from mutations of other genes involved in the regulation of the expression of several genes, including PDGF genes (3). This abnormal local production of PDGF may not interfere with tumor cell replication if these cells do not present PDGF receptor subunits on their surface, but may play a role in the replication of other cells in a paracrine fashion.

Normal epithelial cells do not express the genes coding for PDGF A and B chains or for PDGF receptor α and β subunits (43, 44, 50). In this respect, normal lung epithelial cells do not express PDGF A and B chain genes, although idiopathic pulmonary fibrosis, a chronic disorder of the lung, is characterized by the expression of PDGF B chain gene by hyperplastic type II pneumocytes (43, 44). In lung cancer, PDGF A and B chain gene expression has been observed in several non-small cell lung cancer cell lines (51–53), although two small cell lung cancer cell lines did not express either A or B chain genes (54). One study has addressed the question of PDGF in vivo presence by indirect immunofluorescence, and PDGF protein was detected in 5 of 7 biopsies of non-small cell lung cancer and in 4 of 7 biopsies of small cell lung cancer (53). More recently, an evaluation of 7 non-small cell and small cell primary lung cancers has shown, by in situ hybridization, that all 7 cases were characterized by the expression of PDGF B chain and PDGF receptor β subunit genes, while PDGF A chain and PDGF receptor α subunit gene expression was not evaluated (50). Finally, it has been suggested that PDGF expression by lung cancer cell lines, in association with TGF-α and TGF-β expression, plays a role in tumor stroma formation, since only the cell lines expressing these genes induced a significant tumor stroma when injected to nude mice (55).

The present observation has been limited to NSCLC. Considering the number of tumors studied (n = 64), it seems reasonable to conclude that in NSCLC, the expression by cancer cells of PDGF A and/or B chains is limited to 1 of 3 tumors. Furthermore, if we take into consideration the fact that 1 of 2 tumors expressed PDGF receptor subunits, and only 1 of 6 tumors were characterized by a coexpression of PDGF cytokine and receptor, it follows that an autocrine mechanism involving PDGF in the cancer growth is likely to be limited to a few NSCLC. However, since one-half of tumors express PDGF receptors, the PDGF cytokine could participate to the tumor progression in a paracrine fashion since all cancers contain TAM-producing PDGF A and B chains. This could be particularly true for squamous cell lung carcinomas, in which the higher tumor cell-replicating rates were found in the peripheral part of the tumor in the vicinity of the highest concentrations of PDGF-producing TAM.

Moreover, PDGF cytokine can strongly interfere with the stroma progression. The double source of PDGF (from TAM in almost all tumors and from cancer cells in 1 of 3 tumors), in conjunction with the constant presence of PDGF receptors on mesenchymal and endothelial cells, suggests a possible paracrine involvement of PDGF in NSCLC stroma development. Indeed, the high number of TAM present in tumor stroma and, among them, the high number of PDGF-producing TAM present in the majority of tumors suggests the presence of a relatively high in situ concentration of PDGF. This observation strongly supports a role for TAM in inducing mesenchymal cell and endothelial cell accumulation (by migration and replication) through the presence of PDGF. In this respect, this concept is further supported by the fact that the higher concentrations of PDGF-producing TAM and of replicating endothelial and mesenchymal cells are observed in the same area, namely, the peripheral part of the tumor that is likely to be the site of an ongoing active growing front of tumor extension into the surrounding normal lung tissue. If mesenchymal cell accumulation is important as a solid support for carcinoma cells, the parallel development of new vessels by local replication of endothelial cells is even more central to the possible development of tumor cells. In this respect, the strong expression of PDGF receptor subunits on endothelial cells suggests the role of this cytokine in neovascularization. Interestingly, recent animal data suggests that PDGF BB is directly involved in neovessel formation in nude mice grafted with human WM9 melanoma cells transfected with PDGF-B chain complementary DNA (56).

Several studies suggest that TAM presence in the vicinity of tumor cells is mainly due to the release of specific chemotactic factor(s) by these cells (57–60). Thus, a sequence of events can be proposed to explain tumor stroma formation: (a) recruitment of TAM by the release by tumor cells of chemotactic factor(s) for blood monocytes; and then (b) recruitment and local proliferation of mesenchymal and endothelial cells due to the release of PDGF by TAM and, to a lesser extent, by tumor cells.

This sequence of events can be related to the previous observations made on the succession of cell type accumulation in wound healing and fibrosis. Wound healing is characterized by the early local recruitment of polymorphonuclear neutrophils, followed by the recruitment of blood monocytes (61). These blood monocytes locally transform into tissue macrophages, and their activation results in the local release of several mediators including PDGF (62). The biological activity of PDGF explains the recruitment and local proliferation of mesenchymal cells and their local production of extra cellular matrix, contributing to the processes of repair. Tissue fibrosis has been suggested to result from an excessive process of wound healing, and...
macrophages have been shown to participate in this fibrotic disorder by their excessive local production of PDGF (12, 14, 15). Specifically, previous studies on lung biopsies obtained from patients with idiopathic pulmonary fibrosis showed the strong expression of PDGF B chain gene and production of PDGF by interstitial lung macrophages (43, 44). Thus, the chronic process heading to tumor formation in NSCLC can be related to the mechanisms likely to explain fibrosis of the lung in idiopathic pulmonary fibrosis. In this respect, this study confirms the previously evoked comparison of tumor stroma formation with wound healing in response to a chronic injury (1).

ACKNOWLEDGMENTS

We thank Nathalie Thomas and Michèle Geny for their patient and talented typing.

REFERENCES


The Role of Platelet-derived Growth Factor Production by Tumor-associated Macrophages in Tumor Stroma Formation in Lung Cancer

Jean-Michel Vignaud, Béatrice Marie, Nathalie Klein, et al.


Updated version  Access the most recent version of this article at:  
http://cancerres.aacrjournals.org/content/54/20/5455

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.