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Platelet-derived growth factor (PDGF) in oncogenesis: Development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB

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ABSTRACT **Human WM9 melanoma cells, previously shown to be devoid of PDGF expression, were stably transfected with a PDGF-B cDNA under the transcriptional control of a cytomegalovirus promoter. Northern blot analysis revealed high expression of an mRNA of the expected size in the PDGF-B-transfected cells. Synthesis and secretion of PDGF-BB was confirmed by immunoprecipitation. Furthermore, conditioned medium from PDGF-B-transfected cells contained a mitogenic activity for fibroblasts. For analysis of tumor growth *in vivo*, cells of each type were injected subcutaneously into BALB/c *nu/nu* mice. Tumors from mice injected with WM9 cells transfected with the vector only contained large necrotic areas; only scant blood vessels with narrow lumina were observed. No connective tissue was present. In the tumors from PDGF-B-transfected WM9 cells, nests of tumor were divided by connective tissue septa. An abundance of blood vessels was observed in the connective tissue septa and within the tumor cell nests. There was a complete absence of necrosis in these tumors. The present results suggest that tumor-derived PDGF-BB is a potent mediator of connective tissue stroma formation. The connective tissue framework that is generated in response to PDGF-BB may form a solid support for newly formed blood vessels and, thereby, facilitate the formation of a functional vascular system in the tumor.**

PDGF is a potent mitogen (1, 2) and chemoattractant (3, 4) for fibroblasts. PDGF is a 30-kDa dimer of A and B polypeptide chains linked by disulfide bonds (2, 5). All three isoforms of PDGF (PDGF-AA, PDGF-AB, and PDGF-BB) are products of various normal cells including platelets. Two types of receptors for PDGF have been identified: the α-receptor binds all three isoforms of PDGF with high affinity, whereas the β-receptor only binds PDGF-BB with high affinity (6, 7).

Much of the interest for PDGF in cancer research emanates from the finding that the v-*sis* oncogene of simian sarcoma virus is a retroviral homolog of the cellular gene encoding the B chain of PDGF (8–10). Subsequent studies have provided unequivocal evidence that transformation of cultured cells by the *sis* oncogene is mediated by an autocrine PDGF-BB-like growth factor (for review, see ref. 11). The frequent observation that one or several isoforms of PDGF are coexpressed with the cognate receptor in human sarcoma (12) and glioma cells (13) has been taken as circumstantial evidence for an autocrine mechanism in the development of the corresponding tumor types *in vivo*, although proof for this concept is still lacking (11). However, a number of PDGF-receptor-negative tumor cell lines have also been shown to express PDGF (5), suggesting an alternative (i.e., nonautocrine) role for the growth factor in oncogenesis.

The ability of tumor cells to elicit a stroma reaction is well established. Tumor cells are distinguished from normal cells by their capacity to generate an ingrowth of blood vessels (neoangiogenesis) and this capacity is required for the rapid growth of the tumor (14, 15). Tumor angiogenesis is mediated by specific factors, such as basic fibroblast growth factor, that elicit a profuse capillary ingrowth (15).

In comparison with the neovascular system, very little attention has been paid to the connective tissue stroma component of malignant tumors. In analogy with what is known about the mechanism of tumor angiogenesis, one might assume that the development of a fibroblastic stroma constitutes the host's response to specific tumor-derived connective tissue growth factors. Indeed, Ehrlich (16) formulated the hypothesis that ". . . the transplanted (tumor) cells exert a direct chemotactic influence on the fibroblasts of the host." In the present study we have asked whether PDGF is one of the factors that elicit a stroma reaction when released by tumor cells. Human WM9 melanoma cells, previously shown to be devoid of endogenous PDGF production (17), were stably transfected with a PDGF-B cDNA expression vector and xenotransplanted to athymic *nu/nu* mice. Cells transfected with the vector alone gave rise to necrotizing tumors that were extremely poor in connective tissue stroma; PDGF-B-expressing cells yielded nonnecrotizing tumors with a prominent network of connective tissue stroma containing an abundance of blood vessels. Our data suggest a role for PDGF in oncogenesis as an inducer of a connective tissue stroma that forms a supportive framework for the newly formed tumor vascular system.

MATERIALS AND METHODS

Construction of the Cytomegalovirus (CMV)–PDGF-B Expression Vector. A PDGF cDNA fragment corresponding to the minimal coding sequence, lacking both the 5′ and 3′ untranslated sequences, was amplified by the polymerase chain reaction (PCR) using a human PDGF-B cDNA clone (C. Bethsholtz, personal communication) as a template. The 5′ primer was a 30-base-pair oligonucleotide (5′-AGTCTAGACCATGAATCGCTGCTGGGCGCT-3′). The 3′ primer was a 28-base-pair oligonucleotide (5′-TCGGATCCATGCCCCTAGGCTCCAAGGG-3′). The resulting 0.7-kilobase (kb) fragment was cloned into M13 (Kabi-Pharmacia, Uppsala, Sweden) and sequenced (18); it was then inserted as a *Hin*dIII–*Bam*HI fragment into the multiple cloning site of the pcDNA1/NEO vector (Invitrogen, San Diego) downstream of the CMV promoter.

Transfection of WM9 Cells. The melanoma cell line WM9 (19) was grown and maintained in Eagle's minimum essential

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Abbreviations: PDGF, platelet-derived growth factor; CMV, cytomegalovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
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medium supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 units/ml), and streptomycin (50 μg/ml). Subconfluent cell cultures were transfected with 40 μg of plasmid using the Lipofectin reagent kit (GIBCO/BRL). After culturing the transfected cells in Geniticin (G418; GIBCO/BRL) for 2 weeks, resistant clones were mass-selected and carried as a cell line (WM9/PDGF-B or WM9/vec). Frozen stocks of the transfected cells were kept in liquid nitrogen. The transfected WM9 cells were continuously cultivated in G418.

Extraction of mRNA and Northern Blot Analysis. Total cellular RNA was extracted from confluent cell cultures using the LiCl/urea method (20). Samples for Northern blot hybridization were electrophoresed on 2.2 M formaldehyde gels using 15 μg of total RNA per lane. Gels were blotted onto nitrocellulose-coated nylon filters (Hybond; Amersham). The ^{32}P-labeled probe for detection of PDGF transcripts was prepared using a commercial kit for random priming (Amersham). Hybridization and washing of filters were carried out under high-stringency conditions after which the filters were exposed to Kodak XAR5 films for 1–14 days.

DNA Probes. The PDGF-B PCR fragment described above was used as a probe for Northern blot hybridization. A PDGF-A cDNA probe was prepared by the same method. PDGF α-receptor and PDGFR β-receptor probes were kindly provided by L. Welsh (Ludwig Institute for Cancer Research, Uppsala). The plasmid pHcGAP3 containing cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was a gift from R. Wu (Cornell University, Ithica, NY).

Immunoprecipitation Analysis. Subconfluent cultures of WM9/vec and WM9/PDGF-B cells grown on 50-mm dishes were metabolically labeled for 16 hr with [^{35}S]cysteine (Amersham; 0.1 mCi/ml; 1 Ci = 37 GBq) in serum-free MCDB 104 medium. After labeling, immunoprecipitation of medium and cell lysates was performed as described (21) using a rabbit anti-PDGF antiserum (22). Samples were analyzed by SDS/gel electrophoresis using a 12–18% polyacrylamide gradient gel. The gel was fixed, soaked in Amplify (Amersham), dried, and subjected to flurography using Fuji RX films for 1 week.

Assay of Mitogenic Activity. To examine whether the WM9/PDGF-B cells secreted a mitogenic activity, serum-free MCDB 104 medium conditioned for 72 hr by subconfluent cultures of PDGF-transfected cultures was tested in a [^{3}H]thymidine incorporation assay using normal human fibroblasts as described (23). Medium from WM9/vec cultures was included as a control.

Tumorigenicity in Nude Mice. Two million tumor cells in 50 μl of Hank's balanced salt solution were injected subcutaneously in the backs of 4- to 6-week-old female BALB/c *nu/nu* mice. Five mice were inoculated with WM9/PDGF-B cells; the same number of animals received WM9/vec cells. Three-dimensional measurement of tumors was carried out every week, and tumor volume was expressed in cm^3. Animals were euthanized when their tumors reached ≈3 cm^3.

Histology. Subcutaneously growing tumors and the overlying skin were removed. Half of the tissue was fixed in 10% (vol/vol) neutral formalin and embedded in paraffin. Subsequently, 5-μm sections were prepared and stained with hematoxylin and eosin or Gomori's trichrome. Alternatively, tissue samples were embedded in OCT compound (Miles), snap-frozen, and stored at −70°C until 4- to 6-μm frozen sections were prepared.

Visualization of Endothelial Cells by Lectin Binding. Unfixed frozen sections of tumors were incubated with biotinylated *Griffonia simplicifolia* lectin I (isolectin B4, binding specifically to murine endothelium; Vector Laboratories) at 10 μg/ml in phosphate-buffered saline (PBS) for 1 hr at room temperature. After washing the slides for three 10-min periods in PBS, samples were incubated with fluorescein isothiocyanate-conjugated streptavidin (Jackson ImmunoResearch). After final washings, coverslips were mounted on slides and slides were examined using a Leitz microscope equipped for epiflourescence.

RESULTS

Expression of PDGF-B in WM9 Cells. We constructed an expression vector that utilizes only the protein-coding part of the PDGF-B cDNA (for details of PDGF-B/c-*sis* coding sequences, see ref. 24). Thus, we omitted the entire 5′ untranslated region (≈1 kb) that has been shown (25) to negatively influence translation of the PDGF-B transcript. We also deleted the 3′ untranslated sequences (≈1.6 kb), although there is no direct evidence that this part of the gene has a negative influence on expression levels. However, the first 120 nucleotides of the 3′ end of exon 7 are highly conserved in the human, murine, and feline species (26) and contain one copy of the sequence AUUUA, implicated in mediating RNA degradation (27). Moreover, the most 5′ 149 base pairs of exon 7 are deleted in v-*sis* (8, 24); the same gene segment is apparently also selected against in rat fibrosarcomas induced by an artificial PDGF-B retrovirus construct (28). Transcription from the expression vector (Fig. 1) would yield an mRNA of ≈0.7 kb and thus be readily distinguishable from any endogenous PDGF-B/c-*sis* transcript (3.8 kb) (24, 29).

Northern blot analysis of the PDGF-B-transfected WM9 cells (WM9-PDGF-B) showed a transcript of the expected size (Fig. 2); no signal was obtained using RNA from cells transfected with the vector only (WM9-vec). Hybridization to the probe for GAPDH showed that an approximately equal amount of RNA had been loaded on the gel. Neither of the cell lines contained detectable amounts of PDGF-A, PDGF α-receptor, or PDGF β-receptor mRNA (data not shown). These results are compatible with previous findings (15).

Immunoprecipitation of PDGF-BB from Transfected WM9 Cells. Transfected cells were metabolically labeled with [^{35}S]cysteine after which cell lysates and media were immunoprecipitated using rabbit anti-PDGF antibodies. Fig. 3 shows the autoradiogram of the SDS/polyacrylamide gel of the immunoprecipitates. Under nonreducing conditions, components of 30 kDa and 32–34 kDa were identified in the conditioned medium of WM9-PDGF-B cells; these species were converted to 18 kDa under reducing conditions (lane 2). A small amount of a 24-kDa component was precipitated from the WM9-PDGF-B cell lysate (lane 5); reduction of this component yielded species of 15 kDa (lane 1). Conditioned media or lysates of the WM9-vec cells yielded no specifically precipitated components (lanes 3, 4, 7, and 8).

PDGF-B-Transfected WM9 Cells Secrete a Mitogenic Activity. Conditioned medium from WM9 cells transfected with the PDGF-B expression plasmid or the vector alone was tested in a mitogenic assay using normal human fibroblasts. Fig. 4 shows that the addition of 6% of medium conditioned by WM9-PDGF-B cells for 72 hr stimulated thymidine incorporation into AG1523 cells to the same extent as the addition

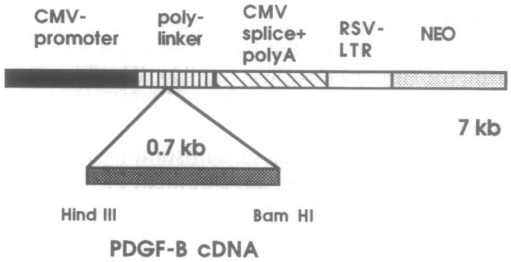

FIG. 1. PDGF-B expression vector. PDGF-B cDNA was inserted as a 0.7-kb *Hin*dIII–*Bam*HI fragment behind the CMV promoter. Splice and polyadenylylation signals from CMV provide correct processing of the mRNA. The gene for neomycin resistance driven by a Rous sarcoma virus long terminal repeat (RSV–LTR) was used as a selection marker.

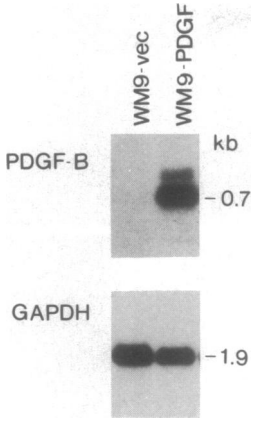

FIG. 2. Northern blot analysis of total RNA extracted from WM9 cells transfected with PDGF (WM9/PDGF) or the vector only (WM9/vec). Hybridization using a PDGF-B probe revealed a 0.7-kb transcript corresponding to the transfected PDGF. Hybridization with a probe for GAPDH showed an even loading of RNA on the gel.

of PDGF-BB (10 ng/ml), used as a positive control. Addition of conditioned control medium (from WM9-vec cells) only marginally increased the [^{3}H]thymidine incorporation of the fibroblasts.

Tumorigenicity in Nude Mice. For the analysis of *in vivo* tumor growth, 2×10^6 cells of each cell type (WM9-PDGF-B and WM9-vec) were injected into nude mice, five animals per group. All mice injected showed tumor growth. Tumors in animals inoculated with WM9-vec cells appeared ≈2 weeks later than in mice injected with PDGF-B-transfected cells. After the tumors had appeared, no differences in their growth dynamics could be observed (Fig. 5).

Morphological Analysis of Tumors. Tumors from mice inoculated with PDGF-B-transfected cells (WM9-PDGF-B) presented as firm grayish-yellow masses. Macroscopic evaluation of cut surfaces revealed no evidence of tissue degradation. Conversely, tumors of similar size from mock-transfected cells (WM9-vec) showed large areas of necrosis.

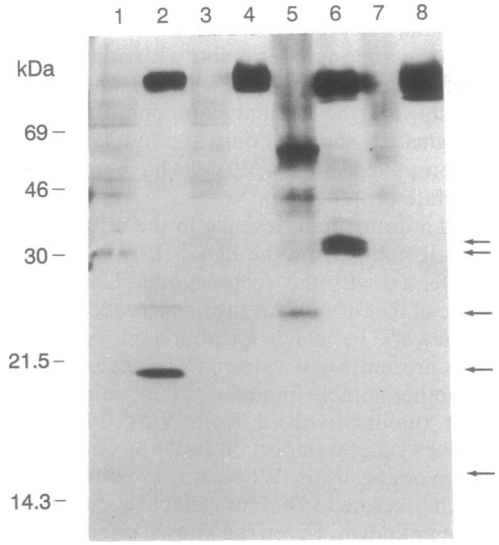

FIG. 3. Immunoprecipitation of metabolically labeled transfected WM9 cells with an antiserum to PDGF. Samples were analyzed electrophoretically under reducing (lanes 1–4) or nonreducing (lanes 5–8) conditions. Lanes: 1, dithiothreitol (DTT)-treated cell lysate (WM9/PDGF-B); 2, DTT-treated medium (WM9/PDGF-B); 3, DTT-treated cell lysate (WM9/vec); 4, DTT-treated medium (WM9/vec); 5, cell lysate (WM9/PDGF-B); 6, medium (WM9/PDGF-B); 7, cell lysate (WM9/vec); 8, medium (WM9/vec).

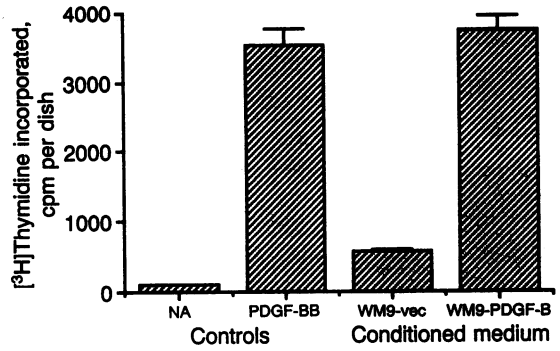

FIG. 4. Assay for mitogenic activity secreted by the transfected WM9 cells. Duplicate dishes of quiescent AG1523 cells received 6% (150 μl) of the total culture volume of medium conditioned for 3 days by WM9/PDGF-B cells or WM9/vec control cells with [^{3}H]thymidine. Parallel cell cultures received PDGF-BB (10 ng/ml) or no addition (NA). The cultures were harvested after 48 hr and radioactivity was measured by liquid scintillation counting.

Histologically, tumors from WM9-PDGF-B cells contained nests of tumor cells divided by septa of connective tissue (Fig. 6*A*). Cells within such nests showed homogeneous growth with many mitotic figures. An abundance of blood vessels was observed primarily below the tumor capsule and in association with collagen septa dividing individual tumor cell nests. Blood vessels were also conspicuous within tumor nests, central to the surrounding connective tissue (Fig. 6 *A* and *C*). Association of blood vessels with the tumor capsule and connective tissue septa became more obvious when frozen sections of tumors were stained with biotinylated *Griffonia simplicifolia* lectin that specifically binds to murine endothelial cells (Fig. 7*A*). Similar to this, abundant blood vessels were observed within tumor nests (Fig. 7*B*).

Tumors from WM9-vec cells were surrounded by a very thin capsule but did not contain connective tissue within the tumor mass (Fig. 6*B*). Only few blood vessels with narrow lumina could be observed. These tumors contained large necrotic areas (Fig. 6*D*). The presence of a number of foci of polymorphonuclear leukocytes also indicated extensive tissue degradation (Fig. 6*D*). Lectin staining for endothelial cells revealed only a few small blood vessels showing no specific pattern of organization (Fig. 7*C*).

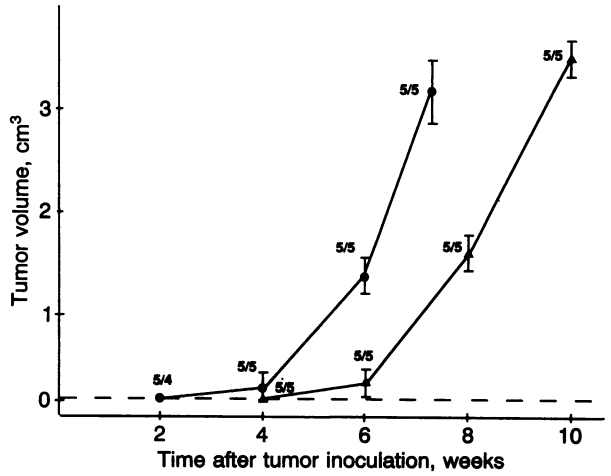

FIG. 5. Tumorigenicity of PDGF-B-transfected and mock-transfected WM9 melanoma cells after subcutaneous injection (2×10^6 cells per 50 μl) in nude mice. All mice presented with tumors. Tumors from mock-transfected cells appeared ≈2 weeks later than those arising from PDGF-B-transfected cells. Note that there is no major difference in growth dynamics between the two groups. ●, WM9-PDGF-B; ▲, WM9-CMV.

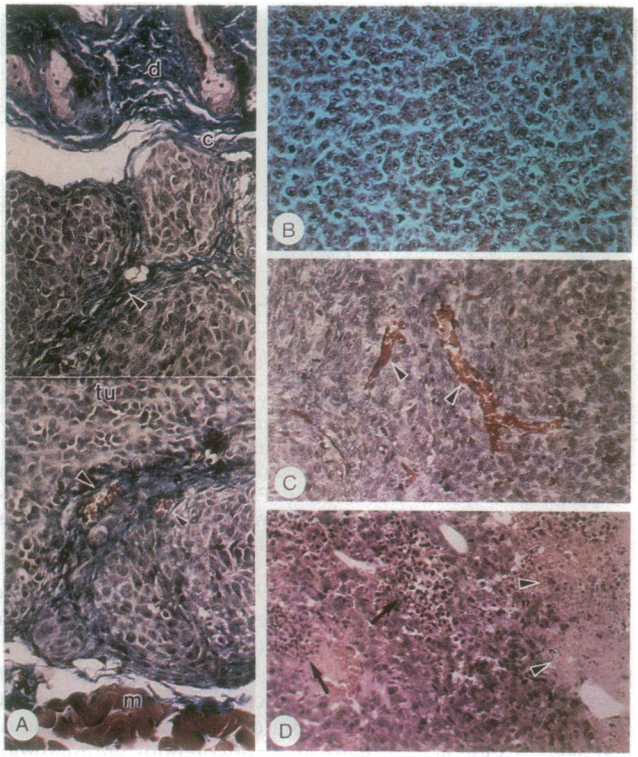

FIG. 6. Histological features of subcutaneously growing tumors from PDGF-B-transfected and mock-transfected WM9 cells. (*A*) Gomori's trichrome staining of tumor tissue derived from injected WM9/PDGF-B cells. Blue staining detects dermal collagen (d), tumor capsule (c), and collagen trabeculae dividing tumor cell nests. Blood vessels (arrowheads) are associated primarily with collagen septae within the tumor (tu) (m, muscular layer). (*B*) Gomori's trichrome staining of tumor tissue derived from WM9-PDGF-vec cells. No connective tissue is detected. (*C*) Hematoxylin/eosin staining of WM9/PDGF-B tumor tissue showing abundant vascular beds with widely open lumina (arrowheads). (*D*) Hematoxylin/eosin staining of WM9-vec tumor tissue showing large areas of necroses (arrowheads) and infiltrates of polymorhponuclear leukocytes (arrows).

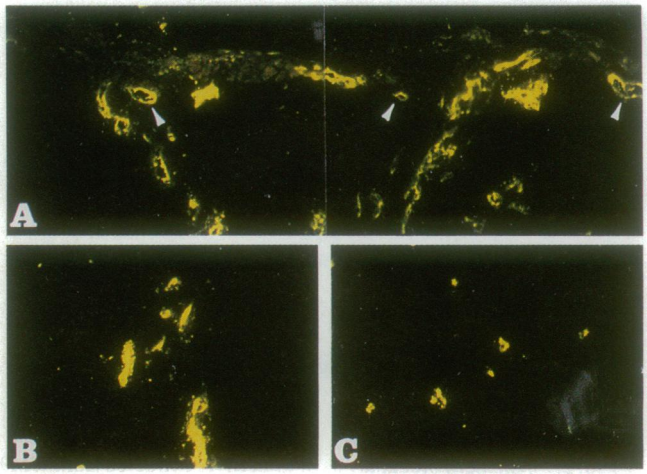

FIG. 7. Detection of blood vessels with *Griffonia simplicifolia* lectin in tumors from PDGF-B-transfected and mock-transfected WM9 cells. (*A*) WM9/PDGF-B tumor tissue. Large blood vessels with open lumina are located in close proximity to the tumor capsule (arrowheads) or associated with collagen septae dividing tumor nests. (*B*) Higher magnification of the tissue shown in *A* detects abundant blood vessels also in the center of tumor nests. (*C*) WM9/vec tumor tissue. Blood vessels with small lumina grow in a random fashion.

DISCUSSION

The stroma is an important component of solid tumors as it has vital functions in supporting the tumor and in providing nutrients and gas exchange over distances not covered by simple diffusion processes (for review, see ref. 30). Tumor stroma is mainly composed of newly formed blood vessels and a connective tissue framework. The mechanism by which the tumor stroma is induced is essentially unknown. In the present study we examined the effect on stroma development of PDGF produced by xenotransplanted WM9 melanoma cells stably transfected with a PDGF-B/c-*sis* expression vector.

The expression of the transduced PDGF-B chain in WM9 cells was characterized by a series of *in vitro* experiments. Northern blot analysis revealed a strong expression of a PDGF-B mRNA of the expected size in the PDGF-B-transfected cells. No endogenous PDGF mRNA was detected, which is in line with our previous report (17).

The synthesis and secretion of PDGF-BB was further verified by testing conditioned medium from WM9/PDGF-B cells in a mitogenic assay and by immunoprecipitation. Metabolically labeled WM9/PDGF-B cells showed the presence in the medium of two forms of PDGF-BB, at 30 kDa and 32–34 kDa, and a small amount of an intracellular 24-kDa species. This finding differs from previous studies of PDGF-B-expressing cells that have identified the cell-associated 24-kDa product as the predominant processed form of PDGF-BB (21, 31); in some cell types, small amounts of a 30-kDa secreted form have been found (21, 32–34). Recent data have indicated that cell association of PDGF-BB is mediated by an amino acid sequence motif in the C-terminal propeptide of the B chain (35) that targets newly synthesized PDGF-BB to an intracellular pathway, where it is proteolytically processed to 24 kDa and finally degraded (34). An alternative pathway, where a 34-kDa PDGF-BB is anchored by the retention signal to an unidentified structure at the cell surface of *sis*-transformed NIH 3T3 cells has been suggested (36). Recently, two reports (37, 38) suggest that a sequence within exon 6 confers binding of PDGF to heparan sulfate proteoglycan associated with the cell or the extracellular matrix. This could result in a localized high concentration of the growth factor. Thus, in some cell types PDGF-BB is processed mainly for autocrine stimulation and in others it is processed for paracrine interaction with adjacent cells. The presence of a secreted form of PDGF-BB in WM9-PDGF-B cells suggests that the cellular component recognizing the retention signal is expressed only at a low level in the WM9 cells or, alternatively, that WM9 cells have only low levels of heparan sulfate proteoglycan.

We found a dramatic difference in the stroma component of tumors originating from the PDGF-B-transfected cells vs. those transfected with the vector alone. The most conspicuous feature of the former was the occurrence of a connective tissue framework in which an abundant system of blood vessels was present; such a stroma was absent in the control tumors. Another notable finding was the complete absence of necrosis in tumors evolved from WM9-PDGF-BB cells. These findings suggest important functions of tumor-derived PDGF-BB in oncogenesis. Whether these effects are directly caused by the secreted PDGF or reflect secondary processes is at present unknown. Given the known effects of PDGF-BB on fibroblasts in culture, it is not unlikely that the connective tissue component of the stroma is formed by fibroblasts that respond by chemotaxis, growth, and matrix production to PDGF secreted by the tumor cells. This idea does not preclude the possibility that infiltrating inflammatory cells contribute to the stroma response. Both neutrophils and monocytes respond chemotactically to PDGF (4, 39). However, accumulation of such cells was not a prominent feature

of the WM9-PDGF-B tumors, and their contribution to the stroma response may be only marginal.

Another important question concerns the role of PDGF on the development of the vascular component of the tumor stroma. Routine histology and staining of endothelial cells using *Griffonia* lectin revealed an increased abundance of blood vessels and a different configuration of the vascular system in tumors generated by the WM9-PDGF-B cells, as compared to the mock-transfected cells (WM9-PDGF-vec). There is recent evidence that microvascular endothelial cells have PDGF β-receptors (40, 41) or α-receptors (42) and respond to PDGF by an increased proliferation rate (41, 43), but whether PDGF can elicit an angiogenic response on its own is still unknown. One must also consider the possibility that PDGF may contribute to neovascularization by syngergizing with a bona fide angiogenic factor released by the tumor cells. Another interesting possibility is that the enhanced angiogenic response is caused by the connective tissue stroma, in that it forms a structural framework for the newly formed vessels.

The occurrence of necrosis is a common event in malignant tumors. A regional insufficiency in the circulation, resulting in necrosis, may obviously be attributed to an incomplete neovascularization but probably equally important is the relatively high interstitial pressure that often prevails in tumor tissue (for review, see ref. 44). This may lead to vascular collapse, complete cessation of regional microcirculation, and ensuing necrosis. In the present study, we found prominent necrosis in tumors derived from mock-transfected WM9 cells, as observed for xenotransplanted wild-type WM9 cells (45). The complete absence of necrosis in the WM9-PDGF-B tumors indicates that PDGF mediates the development of a stroma that fully accommodates metabolic requirements of the growing tumor. It is possible that the connective tissue framework of the tumors forms a mechanical support for the blood vessels that prevents them from collapsing. Furthermore, the division of the tumor tissue into connective-tissue-encapsuled nodules in which blood vessels run may optimize vascular communication with the central parts of the tumor. It is interesting to speculate that a function of PDGF in oncogenesis, in addition to being an autocrine growth factor, is to cause such an organoid transformation of the tumor tissue. The present study thus shows that genetic manipulation of transplantable tumor cells may provide valuable information on the role of growth factors in tumor growth as well as in the development of an organized tissue structure in a broader sense.

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