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Platelet-derived growth factor induces apoptosis in growth-arrested murine fibroblasts

HYEONG-REH CHOI KIM*, SUNIL UPADHYAY*, GANGYONG LI*, KENNETH C. PALMER*, AND THOMAS F. DEUEL†‡

†Departments of Medicine and Biochemistry and Molecular Biophysics, Washington University School of Medicine, and The Jewish Hospital of St. Louis, 216 South Kingshighway, St. Louis, MO 63110; and *Department of Pathology, Wayne State University School of Medicine, 540 East Canfield Avenue, Detroit, MI 48201

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ABSTRACT **The platelet-derived growth factor (PDGF) is a potent mitogen for murine fibroblasts. PDGF-stimulated cells express a set of immediate-early-response genes but require additional (progression) factors in serum to progress through the cell cycle. Serum-deprived cells are reversibly arrested in G_0 phase and fail to fully traverse the G_1 phase of the cell cycle when stimulated by PDGF alone. We now report that serum-deprived normal rat kidney fibroblast (NRK) cells stimulated by either PDGF AA or PDGF BB homodimers undergo apoptotic cell death. Furthermore, we show that epidermal growth factor also induces apoptotic cell death in serum-deprived NRK cells, epidermal growth factor enhances the rate of apoptosis in PDGF-treated cells, and a progression factor (insulin) but not endogenously expressed Bcl-2 fully protects NRK cells from PDGF-stimulated apoptosis. The results indicate that PDGF induces apoptosis in growth-arrested NRK cells and that the inability of NRK cells to transit the G_1/S checkpoint is the critical determinant in establishing the genetic program(s) to direct the PDGF signal to apoptosis. The results suggest that polypeptide growth factors *in vivo* may signal cell fate positively or negatively in settings that limit the potential of cells to completely transit the cell cycle.**

The platelet-derived growth factor (PDGF) is a potent mitogen *in vitro* (1–3). However, PDGF also signals a diversity of other important cellular responses, including chemotaxis (4–6), survival (7), and transformation (8–12), that correlate with proposed functional roles of PDGF *in vivo* in normal development (13, 14), inflammation (1, 15, 16), and wound healing (17, 18) and in the abnormal cell migration and proliferative responses characteristic of atherosclerosis (1–3), fibrosis (1–3, 19, 20), and neoplasia (1–3, 8–12). Whereas high levels of expression of PDGF have been identified in tissues from each of these processes, the cells within these tissues are often found in areas that are within unfavorable environments for cell growth, raising the important question whether the responses of cells to PDGF may vary with genetic programs that are activated in cells by local extracellular environmental factors.

Structurally, PDGF is composed of three dimeric polypeptides, the homodimers PDGF AA and BB and the heterodimer PDGF AB. The A and B chains of PDGF are ≈50% identical in amino acid sequence and perfectly conserve cysteine residues. PDGF AB is found almost exclusively in megakaryocytes or in platelets, whereas the AA and BB homodimers are differentially expressed in several different cell types at different levels in both development and adulthood (1, 3, 13, 14, 21). The A- and B-chain genes of PDGF are localized to different chromosomes and are differently and independently regulated (1–3). The PDGF isoforms interact differentially with each of two (α and β) independently expressed PDGF receptors, establishing one basis for the diversity of function of the PDGF isoforms. PDGF BB binds with high affinity to the PDGF α and β receptors, whereas PDGF AA interacts effectively only with the PDGF α receptor (22, 23).

Murine fibroblasts respond to serum deprivation by reversible growth arrest in the G_0 phase (24–27). PDGF functions with serum-starved cells as a "competence" factor (28, 29) to stimulate expression of a set of immediate-early-response genes [including c-*myc* (30), c-*fos* (31), and actin (24, 32)] during the G_0/G_1 transition. The mRNA levels of these genes are sharply reduced as cells transit through G_1/S phase (24). For PDGF-stimulated cells to progress beyond late G_1 phase and transit the cell cycle and divide, progression factors in serum such as insulin and insulin-like growth factor 1 are required (27, 33).

In these experiments, cells cultured in 0.1% serum to impose a high degree of cell cycle arrest were tested as a model to determine whether PDGF-stimulated cells respond differently when cell growth is limited by culture in an unfavorable environment for growth. We establish that both PDGF AA and PDGF BB induce apoptotic cell death in normal rat kidney fibroblast (NRK) cells cultured in 0.1% calf serum for 24 hr but not when cells were stimulated with PDGF in cultures with 10% calf serum. We also establish that epidermal growth (EGF) also induces apoptotic cell death when tested with cells under identical conditions and that insulin but not endogenously expressed Bcl-2 rescues cells from PDGF-induced apoptosis.

METHODS

Cell Culture. NRK cells (American Type Culture Collection) were selected because their growth responses of NRK cells to PDGF had been extensively tested (34, 35). Murine fibroblasts also are known to express well-characterized immediate-early genes (24, 30, 31). NRK cells were grown in 10% calf serum/Dulbecco's modified Eagle's medium (DMEM)/10 mM Hepes, pH 7.5, as described (26, 34, 35). To induce cell-cycle arrest, logarithmically growing cells at ≈10% confluence were washed with phosphate-buffered saline and cultured in 0.1% serum/DMEM/10 mM Hepes buffer, pH 7.5, for 24 hr (26). Medium was changed at 48-hr intervals. Cells were counted after trypsinization with a hemocytometer. PDGF AA and PDGF BB homodimers were from Glenn Pierce and William Kenny (Amgen Biologicals). The incorporation of 5-bromodeoxyuridine (BrdUrd) was done and analyzed as described (35) and used to establish saturating concentrations of PDGF AA and PDGF BB between 25 and 50 ng/ml and half-maximum concentrations between 5 and 10 ng/ml in dose–response analysis. Viable cells were established by exclusion of trypan blue.

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Abbreviations: PDGF, platelet-derived growth factor; NRK, normal rat kidney fibroblast; EGF, epidermal growth factor.

‡To whom reprint requests should be addressed.

Microscopic Analysis. Light microscopy was used at ×40 magnification; Polaroid type 667 film was used. For electron microscopy, cells were scraped from control and treated cultures and washed three times with Hanks' balanced salt solution and centrifuged. Cell pellets were resuspended in 10 vol of fresh 1% glutaraldehyde/1.25% paraformaldehyde/0.2 M sodium cacodylate buffer, pH 7.4, and centrifuged. Pellets were fixed overnight at 4°C, washed in cacodylate buffer (pH 7.4), postfixed in 2% aqueous osmium tetroxide for 1 hr, dehydrated through a graded ethanol series, and embedded in epoxy resin. Ultra-thin sections were cut, stained for contrast with urinyl acetate and lead citrate, and photographed. Electron microscopy was done with a Philips EM400 electron microscope.

Preparation of Cytoplasmic DNA. For electrophoresis of DNA, dying cells in supernatants were collected and lysed for 10 min in 100 ml of cold 20 mM Tris·HCl, pH 7.4/10 mM EDTA/0.2% Triton X-100, the nuclei were precipitated, and the DNA was isolated from cytoplasmic fractions (supernatant) and electrophoresed into 1.5% agarose gel.

RESULTS

To investigate the response of growth-arrested cells to PDGF, sparse (≈10% confluency), logarithmically growing NRK cells were cultured in 0.1% serum/DMEM/10 mM Hepes for 24 hr as described (24–26). Under these conditions, cells in the S phase of the cell cycle are <10% of cells cultured in 1% or 10% serum, indicating a high degree of cell cycle arrest (26, 28). The cells in 0.1% serum/DMEM/10 mM Hepes were chronically stimulated (96 hr) with PDGF AA and PDGF BB at saturating (50 ng/ml) or at subsaturating concentrations (10 ng/ml) as determined (35), and cell numbers were determined at 24, 48, and 96 hr.

The cells treated with either PDGF AA or PDGF BB at saturating levels (50 ng/ml) progressively lost viability (Fig. 1*A*). The loss of viability was essentially equal with PDGF AA or BB; in each instance, only ≈10% of the cells remained viable at 96 hr (Fig. 1*A*). After 6–10 days in culture, viable cells were not detected (data not shown). The effect of PDGF is dose dependent. Cells treated with subsaturating concentrations (10 ng/ml) of PDGF AA or PDGF BB increased slowly in number and progressed toward confluence. Cultures contained ≈3×10^5 cells per well at the time of addition of PDGF AA or BB at 10 ng/ml (subsaturating); ≈$11–15 \times 10^5$ cells were seen at 96 hr. NRK cells cultured in 0.1% serum/DMEM/10 mM Hepes alone also grew slowly and progressed toward confluence. An ≈2-fold increase in cell number (≈5×10^5 cells) was seen at 96 hr. In other control experiments, cell growth was vigorous when logarithmically growing NRK cells in 10% serum/DMEM/10 mM Hepes were treated with PDGF AA or PDGF BB at 10 ng/ml and at 50 ng/ml; loss of viability was not seen, indicating that the cells retained full growth potential. NRK cells in 0.1% serum/DMEM/10 mM Hepes treated with PDGF AA or BB at 50 ng/ml were compared at 10% and 70% confluency. Cell death was induced more efficiently at 10% confluency than at 70% confluency, indicating that depletion of essential nutrients was not responsible for loss of cell viability. However, the result also suggested that at higher density secreted soluble factors may rescue cells in 0.1% serum/medium from the loss of cell viability in the presence of PDGF. Cultures were tested at pH 7.2 and pH 7.6 in 0.1% serum/medium with PDGF AA and BB at both 10 and 50 ng/ml, and the growth kinetics were identical as in cultures at pH 7.5. The pH in the medium was tested at 48 hr and varied by <0.1 pH unit, establishing that the PDGF-dependent loss of viability was not due to changes in extracellular pH. Short-term (overnight) viability and the incorporation of BrdUrd into confluent NRK cells were tested in 0.5% serum/DMEM/10 mM Hepes. Consistent with previous dose–response analyses (35), the response of cells was vigorous; maximal (saturating) BrdUrd incorporation was observed at PDGF AA and BB at 20–25 ng/ml; half-maximal uptake was seen between ≈5 and 10 ng/ml (data not shown). BrdUrd incorporation of cells also was tested after 24 hr in 0.1%

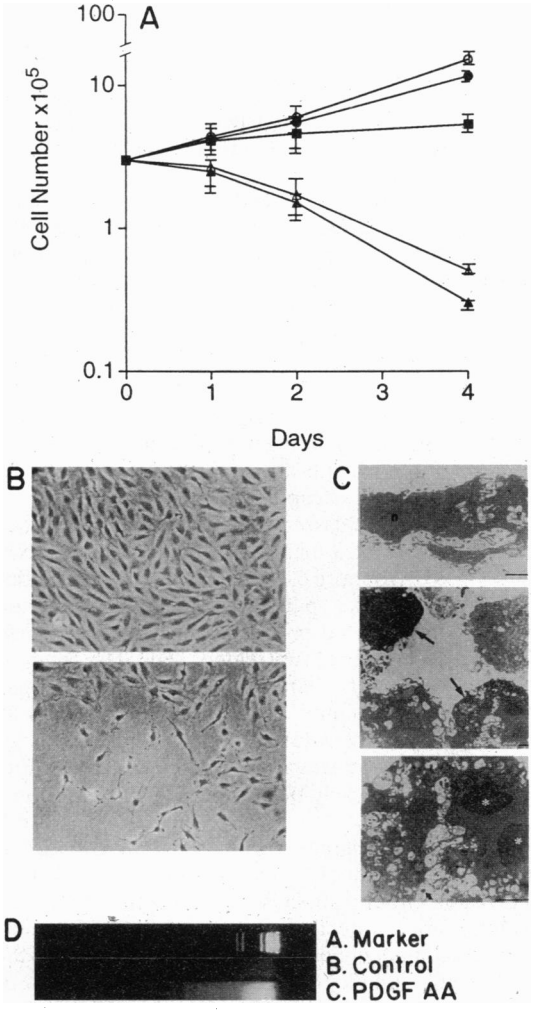

FIG. 1. (*A*) Growth curves of PDGF-treated NRK cells grown in 0.1% serum/DMEM/10 mM Hepes. NRK cells were plated at 10% confluency and cultured as described in 0.1% serum/medium alone (■) or 0.1% serum/medium/PDGF AA (●) or BB (○) at 10 ng/ml or PDGF AA (▲) or BB (△) at 50 ng/ml. Live cells were counted by trypan blue exclusion. Mean values of triplicates are shown plotted against time. (*B*) Effect of PDGF on the growth of NRK cells in 0.1% serum/medium by light microscopy. NRK cells were plated at 20% confluency and treated as in *A*. Cells were maintained (*Upper*) in 0.1% serum/medium alone or (*Lower*) in the presence of PDGF AA (50 ng/ml), and medium was changed every other day. Light microscopy was used to photograph cells after 5 days with or without PDGF AA. (×32.) (*C*) Electron microscopic analysis of NRK cells undergoing apoptosis. NRK cells were treated the same as in *A* with or without PDGF AA at 50 ng/ml for 24 hr and processed for electron microscopy as described. (*Top*) Typical cell from control cultures. The nucleus (n) shows no evidence of degenerative or apoptotic changes and, except for mild dilation of the endoplasmic reticulum, the cytoplasm is unremarkable. (*Middle*) PDGF AA-treated cultures. Of the four cells visible, two (arrows) demonstrate apoptotic changes, including rounded, shrunken profiles, compaction of chromatin, dense masses, and intact lysosomes. (*Bottom*) A high magnification of apoptotic cell from *B*. Chromatin compaction (asterisks) into dense bodies, membrane blebbing, and preservation of intact lysosomes (arrows) are readily appreciated. (Bars = 1.0 μm.) (*D*) DNA fragmentation. Lanes: A, DNA marker; B, cytoplasmic DNA from control culture; C, cytoplasmic DNA from culture treated with PDGF AA homodimers at 50 ng/ml for 48 hr.

serum/medium; <10% BrdUrd incorporation was found in comparison with cells in 0.5%–10% serum/medium and was sufficiently low that assay results were considered unreliable. However, the results served to confirm that culture for 24 hr in 0.1% serum/medium established a high level of growth arrest and that PDGF alone failed to drive cells beyond a G_1/S checkpoint as described (26).

The mechanism by which PDGF-treated cells lose viability was suggested by microscopy. In light microscopy, PDGF-treated cells were reduced in number (Fig. 1*B*, *Lower*) and characteristically demonstrated nuclear condensation, cytoplasmic shrinking, and membrane blebbing that were not seen in control (untreated) cultures. In electron micrographs (Fig. 1*C*), large numbers of treated cells showed the ultrastructural features characteristic of apoptosis. The treated cells were round and shrunken, and condensation of chromatin into multiple dense masses, blebbing of plasma membranes without rupture, and intact, membrane-limited lysosomal structures were observed. In contrast, apart from some cells with minimal expansion of the endoplasmic reticulum, the untreated cells were relatively normal. Furthermore, cytoplasmic DNA was examined by electrophoresis to seek nucleosome laddering (Fig. 1*D*). A "ladder-like" pattern of DNA fragmentation was consistently demonstrated in cells treated with PDGF AA at 50 ng/ml but not in control (untreated) cells. The combination of loss of cell viability, characteristic light microscopic and ultrastructural features, and a DNA "ladder" pattern in gels from cells treated with saturating concentrations of PDGF indicates that PDGF induces apoptotic cell death in cultures of NRK cells with a cell cycle block imposed by growth in 0.1% serum/medium.

PDGF effectively signals early events in the cell cycle, driving cells from G_0 into G_1 phase to establish "competence" for cell division. Other distinct, serum-derived ("progression") factors are required for cells to fully traverse G_1 phase, initiate DNA synthesis, and divide (36–41). To further test the hypothesis that the inability of PDGF-stimulated cells to transit the G_1 checkpoint correlates with the apoptotic response in cells in 0.1% serum/medium, a progression factor (insulin) was added to determine whether progression through the cell cycle would "rescue" the cells from PDGF-dependent apoptotic cell death. This approach proved correct; insulin (10 μg/ml) fully rescued PDGF AA-treated cells (50 ng/ml) cultured in 0.1% serum from apoptotic cell death (Fig. 2*C*) at the same time apoptotic cell death was readily apparent in the control cultures with PDGF AA (50 ng/ml) alone (Fig. 2*B*) but not in control cultures without PDGF (Fig. 2*A*). We conclude from these results that arrest of cells at the G_1 checkpoint in the presence of saturating PDGF concentrations is an essential determinant of the apoptotic response and that progression through the G_1 checkpoint in the presence of insulin effectively prevents the apoptotic response.

To determine whether growth factors other than PDGF induce apoptosis under similar conditions, EGF was tested. EGF alone does not allow entry of serum-starved cells into the S phase of the cell cycle but, in conjunction with other serum factors, sustains progression through the cell cycle and division (27, 37, 38, 42). EGF (50 ng/ml) consistently induced apoptosis when added to NRK cells in 0.1% serum/medium (data not shown). EGF also enhanced the apoptotic response of PDGF AA (50 ng/ml) (Fig. 2*D*). EGF, therefore, also induces apoptotic cell death in growth-arrested NRK cells, enhances the PDGF-dependent apoptotic response, and appears when used in conjunction with PDGF to up-regulate a common signaling molecule that, at higher levels than with PDGF alone, further accelerates the apoptotic pathway. The result also suggests that growth factors, in general, may be important mediators of apoptotic cell death under defined (cell cycle arrest) conditions of culture.

The *bcl-2* gene product blocks the apoptotic cell death that occurs during lymphoid and neural development (43, 44) and is an important regulator of radiation- and calcium ionophore-induced apoptosis in thymocytes and in apoptosis induced by deprivation of growth factors (45–50). However, Bcl-2 is not uniformly effective in preventing apoptotic cell death, indicating that different pathways of apoptotic cell death may be defined by the ability of Bcl-2 to prevent apoptotic cell death (51). We therefore tested clonally selected NRK cells endogenously expressing the *bcl-2* gene driven by the cytomegalovirus promoter (35). In control experiments, 5×10^5 NRK (Fig. 3*A*) and *bcl-2*-containing NRK cells (Fig. 3*B*) were tested with 250 μM hydrogen peroxide for 16 hr. Bcl-2-expressing cells were entirely protected from free-radical-induced apoptosis, whereas NRK cells alone became rapidly apoptotic in hydrogen peroxide. However, *bcl-2*-containing NRK cell clones (1×10^5 cells per plate) in 0.1% serum/PDGF at 50 ng/ml became apoptotic (Fig. 3*D*). PDGF thus induced NRK cell death equally in cells endogenously expressing Bcl-2 (Fig. 3*D*) and in control NRK cells cultured in 0.1% serum/medium (Fig. 2*B*). The untreated cells containing *bcl-2* appeared normal (Fig. 3*C*). This result indicates that PDGF-dependent apoptosis is signaled through a pathway that is not blocked by

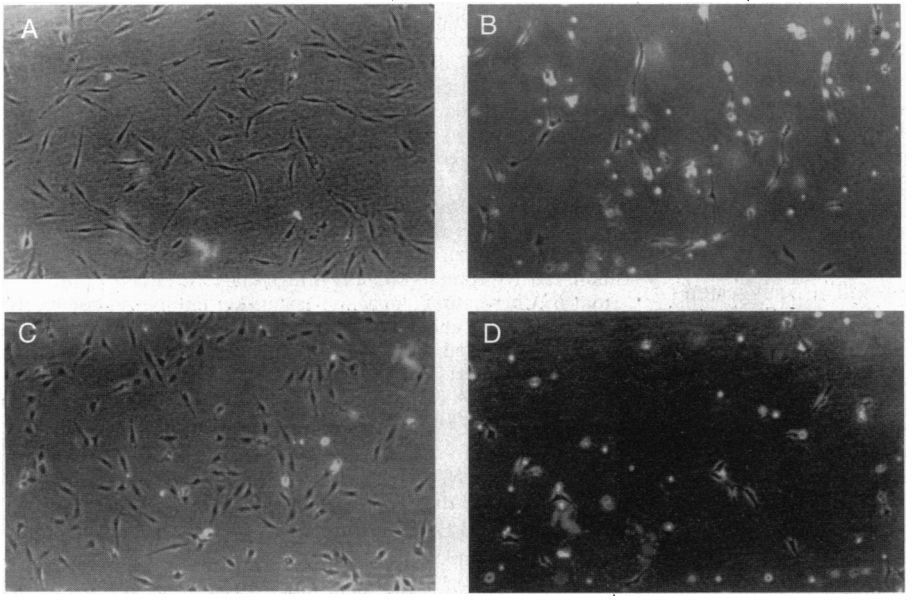

FIG. 2. Rescue by insulin. NRK cells were plated at 10% confluence and treated as for Fig. 1. (*A*) Control. (*B*) NRK cells treated with PDGF AA at 50 ng/ml. (*C*) NRK cells treated with PDGF AA at 50 ng/ml plus insulin at 10 μg/ml. (*D*) NRK cells treated with PDGF AA at 50 ng/ml plus EGF at 50 ng/ml. Cultures were treated for 48 hr and photographed under light microscopy using Polaroid type 667 film. (×32.)

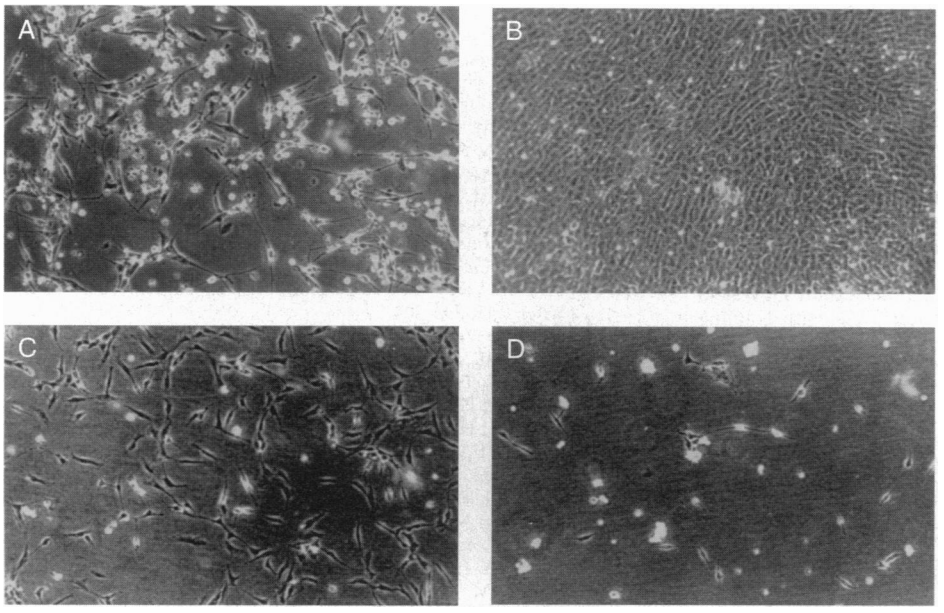

FIG. 3. Bcl-2 fails to block PDGF-induced apoptosis. Control NRK (*A*) or Bcl-2-expressing NRK (*B*) cells (each at 5×10^5 cells per well) were plated in a 6-well plate (near confluence). After culture overnight, 250 μM H_2O_2 was added to the confluent control and Bcl-2-expressing cells. Cultures were photographed on the following day. Bcl-2-expressing NRK cells also were plated at 10^5 cells per T-25 flasks ($\approx$10% confluency). (*C*) Control cells. (*D*) Cells treated with PDGF AA at 50 ng/ml. Cultures were photographed with light microscopy 24 hr after treatment using Polaroid type 667 film. (×32.)

endogenous expression of Bcl-2 in levels that effectively block H_2O_2-induced apoptotic cell death.

DISCUSSION

The molecular mechanisms that regulate apoptotic cell death are only beginning to be discovered. Thus, the recognition that PDGF signals apoptosis and the identification of conditions in which PDGF signals apoptosis are important. This report suggests experimental strategies to investigate the genetic pathways by which PDGF and other growth factors lead to apoptosis and establishes the requirement of cell cycle arrest and inability to transit the G_1/S checkpoint for PDGF-dependent apoptosis. The results also establish the requirement of saturating levels of PDGF and the additive effect of EGF with PDGF in directing cells to apoptosis, suggesting that a common signaling molecule is initiated by PDGF and EGF that functions additively to signal apoptosis in growth-arrested cells. The results separate the PDGF apoptotic pathway from that of oxidant-induced apoptosis and other apoptotic pathways that are blocked by endogenously expressed *bcl-2* gene. The results suggest that it is possible and perhaps likely that PDGF may function in apoptosis *in vivo*. The PDGF concentration in these experiments (50 ng/ml) is found in human serum and is therefore achievable *in vivo*. The results are noteworthy because PDGF is widely recognized as a potent mitogen (1–3) and survival factor (7) and may be of general significance because three independent growth factors (PDGF AA and BB, EGF) signal programmed cell death under identical conditions of a cell cycle block and appear to be additive in triggering apoptosis through independent receptor mechanisms. It is now clear that PDGF initiates both cell growth and death, depending upon the environmental context and therefore the genetic programs extant in target cells.

The conditions that have been identified in this work as essential to the PDGF apoptotic signal are the imposition of G_0 cell cycle arrest and the initiation by PDGF of immediate-early-response signals to drive cells to late G_1 stage. Progression of eukaryotic cells through the cell cycle requires the orderly movement through different cell cycle stages that are highly regulated at checkpoints. The checkpoints function to ensure that cells do not advance until the metabolic events that are needed for progression to the next stage of the cell cycle have been completed (52–54). The tumor-suppressor gene product p53 is an important factor that regulates the G_1 checkpoint before the initiation of DNA synthesis (55). When DNA is damaged, levels of p53 rise, and entry into the S phase is delayed pending repair (56–58). The elevated levels of p53 inhibit cell division (56, 59–61) or result in apoptosis (62–67). Cells with loss of p53 function progress without DNA repair (68, 69) and are associated with genomic instability and tumorigenesis, suggesting an essential function of p53 is to permit DNA repair by G_1 arrest (58) or to signal apoptosis, depending upon cell type and other factors (64, 65). When Rat-1 cells are similarly cultured in 0.1% serum, endogenously expressed c-*myc* (26) or transcription factor E2F (70) also signals apoptosis, suggesting the general view that cells unable to transit cell cycle checkpoints acquire genetic programs that direct apoptosis in response to a number of factors that normally are associated with a growth response. Because c-*myc* is among the immediate-early-response genes that are up-regulated by PDGF and is itself a protooncogene (30), c-*myc* may be an important determinant of the PDGF apoptotic response. The transcription factor E2F activates genetic programs that are required for the synthesis of DNA precursors required for the S phase of the cell cycle (70). When the E2F-1-encoding gene is introduced into cells that contain a temperature-sensitive p53 allele, coexpression of the wild-type p53 protein and transcription factor E2F-1 results in apoptosis, suggesting that when the E2F-1 signal to enter the S phase confronts a p53-imposed delay of entry into S phase, p53 and E2F-1 together signal apoptosis (71) and also suggesting a parallel with PDGF, in which a potent growth signal in the presence of an imposed cell cycle block initiates apoptosis.

Another important mediator in the PDGF-mediated apoptotic response may be p21 [wild-type p53-activated fragment 1 (WAF1) (72); cdk-interacting protein (CIP1) (73)]. p21 is up-regulated by p53, inhibits cyclin-dependent kinases (73–75), and inhibits growth of tumor cell lines (72) and normal diploid fibroblasts (73), suggesting a relationship between p53, G_1 arrest, and negative regulation of the cell cycle kinases required to transit the G_1–S checkpoint (76). Furthermore, p21 is induced by DNA-damaging agents that lead to G_1 arrest or to apoptosis but not in cells with mutant p53 (76), suggesting that the p21 gene product is a downstream mediator of p53 function (72, 73, 77). Importantly, p21 is induced by serum or by purified growth factors, including PDGF.

Our results thus suggest important insights into a potentially highly important role of PDGF in regulating cell growth and death. This role may be of major importance in normal development and in both normal and abnormal proliferative states. These insights into other roles of PDGF may be

important in developing strategies to regulate normal and abnormal proliferative states and to identify additional genes that function in the pathways leading to apoptosis. The results also establish a system in which the regulation of apoptotic-associated genes may be directly analyzed in the presence of a single, well characterized growth molecule.

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