Journal of Cellular Biochemistry 70:70–83 (1998)

Transforming Growth Factor-β1 Induces Apoptotic Cell Death in Cultured Retinal Endothelial Cells But Not Pericytes: Association With Decreased Expression of p21$^{waf1/cip1}$

Qi Yan and E. Helene Sage*

Department of Biological Structure, University of Washington, Seattle, Washington 98195-7420

Abstract Transforming growth factor-β1 (TGF-β1) regulates a variety of cellular functions. In several types of cells, for example, it acts as a growth inhibitor and an inducer of apoptotic cell death. Although one of the important modulators in retinal vascular development and retinal neovascularization, the effects of TGF-β1 on retinal microvascular cells are not fully defined. We have found that proliferation of both bovine retinal endothelial cells (EC) and pericytes was inhibited by TGF-β1 in a concentration-dependent manner. However, only retinal EC lost viability after exposure to increasing concentrations of TGF-β1 (up to 10 μg/ml) in the presence of 2% fetal bovine serum. Dying EC exhibited the morphological and biochemical characteristics of apoptosis. Fragmented nuclei and chromatin condensation were apparent after staining with the fluorochrome Hoechst 33258 and the reagent ApopTag; moreover, gel electrophoresis of DNA from TGF-β1-treated EC demonstrated degradation of chromatin into the discrete fragments typically associated with apoptosis. The addition of anti-TGF-β1 neutralizing antibody abolished the apoptotic cell death induced by TGF-β1. Because not all the EC in a given culture died after exposure to TGF-β1, we separated the apoptosis-sensitive cells from those resistant to TGF-β1-mediated apoptosis and determined the expression of several proteins associated with this apoptotic pathway. Apoptosis of EC mediated by TGF-β1 was associated with a decreased level of the cyclin-dependent kinase inhibitor p21$^{waf1/cip1}$, compared with that observed in the apoptosis-resistant cells. In contrast, the translation product of the tumor-suppressor gene p53 was increased in the TGF-β1-treated apoptotic cells. Thus, we propose that p21$^{waf1/cip1}$ and p53 function in distinct pathways that are protective or permissive, respectively, for the apoptotic signals mediated by TGF-β1. J. Cell. Biochem. 70:70–83, 1998.

Key words: TGF-β1; apoptosis; growth inhibition; retina; endothelial cells; pericytes; angiogenesis; p21$^{waf1/cip1}$; p53

Retinal angiogenesis plays a pivotal role in the development of retinal vasculature and pathological retinal neovascularization. The process of retinal angiogenesis is regulated through stimulatory and inhibitory signals that include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming growth factor-β1 (TGF-β1) [D'Amore, 1994]. VEGF and bFGF are considered to be promoters of angiogenesis that regulate endothelial cell survival, proliferation, migration, and extracellular proteolytic activities [Alon et al., 1995; Montesano et al., 1986; Stone and Maslim, 1997]. In contrast, the role of TGF-β1 in angiogenesis is more complicated and thus has been controversial: TGF-β1 has been described as angiogenic or anti-angiogenic, in vivo and in vitro [Madri et al., 1988; Phillips et al., 1993; Pierce et al., 1995]. A current hypothesis is that the positive or negative regulation of angiogenesis by TGF-β1 depends on the context of the experimental model or the stage of the angiogenic process under study [Pepper, 1997]. TGF-β1 is nevertheless a critical factor in vascular development because TGF-β1-deficient mice have defective vasculogenesis that results in part in abnormal capillary structure [Dickson et al., 1995].

The effects of TGF-β1 on retinal angiogenesis are not entirely clear. Retinal microvasculature

Contract grant sponsor: National Institutes of Health; Contract grant numbers: GM40711 and EY04536; Contract grant sponsor: NIGMS; Contract grant number: PHS NRSA T32 GM07270; Contract grant sponsor: NEI; Contract grant number: T32 EY07031.

*Correspondence to: E. Helene Sage, Department of Biological Structure, Box 357420, University of Washington, Seattle, WA 98195-7420.

Received 8 January 1998; Accepted 2 February 1998

is composed of endothelial cells (EC) and pericytes. Interactions between these cells affect retinal neovascularization, a sequel common to diabetic retinopathy, retinopathy of prematurity, and other ocular retinopathies. TGF-β1 is synthesized by most cells in a latent form that requires activation for interaction with cell-surface receptors. The mechanisms of activation of latent TGF-β1 in vivo are not well understood; however, studies in vitro have shown that interaction between EC and pericytes or smooth muscle cells leads to the activation of secreted TGF-β1 [Antonelli-Orlidge et al., 1989]. In mammals, the retinal microvasculature contains the highest number of pericytes [Kohner et al., 1991; Shepro and Morel, 1993]. Therefore, TGF-β1, secreted locally by retinal microvascular cells, may affect the function of retinal capillary EC and pericytes. This cytokine might maintain vascular quiescence under normal conditions because it inhibits EC proliferation and migration [Roberts and Sporn, 1989]. Moreover, studies in vivo have demonstrated that TGF-β is localized in the retinal ganglion cell layer and pigment epithelium [Lutty et al., 1993; Anderson et al., 1995]; further, elevated levels of TGF-β have been detected in vitreous aspirates from patients with proliferative diabetic retinopathy [Gaudric et al., 1990; Esser et al., 1997]. These and other studies [Orlidge and D'Amore, 1987; Sato and Rifkin, 1989; Tanihara et al., 1997] have provided convincing evidence that TGF-β1 regulates certain functions of retinal EC and pericytes and is likely to mediate selected aspects of retinal neovascularization.

TGF-β1 is a 25-kDa homodimeric polypeptide that exerts pleiotropic effects on cellular proliferation, cellular differentiation, and extracellular matrix production [Kingsley, 1994]. The actions of TGF-β are clearly multifocal and depend on the target cell type, its associated substrate, and the presence of other growth factors. TGF-β1 inhibits cell proliferation, including that of EC and pericytes, in the G1 phase of the cell cycle. Recent interest has been focused on the role of TGF-β1 in cell death. For example, TGF-β1 induced apoptosis in cultured human umbilical vein EC [Tsukada et al., 1995], bovine glomerular capillary EC [Choi and Ballermann, 1995], bovine adrenal microvascular EC [Mandriota and Pepper, 1997], hepatocytes [Fabregat et al., 1996], uterine epithelial cells [Rotello et al., 1991], and several other types of cultured cells [Selvakumaran et al., 1994; Ilio et al., 1995; Lomo et al., 1995; Landstrom et al., 1996; Yamamoto et al., 1996; Ohta et al., 1997]. However, the mechanisms of TGF-β1-induced apoptosis among the different cell types appear to be dissimilar, although data on this point are limited.

Because TGF-β1 is considered to be an integral cytokine in the retinal vasculature and endothelial apoptosis may play a significant role in retinal neovascularization, we asked whether TGF-β1 induces apoptotic death in cells cultured from the retinal microvasculature. We present evidence that TGF-β1 inhibits the proliferation of both retinal EC and pericytes. However, TGF-β1 induced specifically apoptotic cell death in retinal EC but not in pericytes in a concentration-dependent manner. Furthermore, apoptosis of retinal EC was associated with decreased expression of the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$, whereas the product of the tumor suppressor gene p53 was augmented in apoptotic EC, relative to levels in nonapoptotic cells. Thus, $p21^{waf1/cip1}$ and p53 may act on distinct pathways that mediate the inhibition or stimulation, respectively, of apoptosis by TGF-β1.

MATERIALS AND METHODS

Preparation of Cells

Bovine retinal capillary EC were isolated and cultured essentially according to previously described methods, with some modifications for primary cultures [Yan et al., 1996]. Briefly, the minced tissue fragments were digested in 0.3% collagenase/dispase (Boehringer Mannheim Biochemical, Indianapolis, IN) containing 10 U/ml DNase I (Sigma, St. Louis, MO) at 37°C with gentle agitation for 30 min to 1 h. The fragments were washed with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT), and were filtered through a 40-μm nylon sieve (Becton Dickinson, Franklin Lakes, NJ). The fragments on the sieve were collected, resuspended in DMEM with 10% FBS, and plated onto dishes precoated with 20 μg/ml bovine fibronectin (FN; Sigma) for 2–3 h to allow attachment of capillary fragments to the dishes. The medium was subsequently aspirated, and the dishes were rinsed gently with DMEM containing 10% FBS to remove debris, unattached cells, and noncapillary fragments. A complete culture medium was added, and EC were grown in DMEM con-

taining 10% FBS, 10 µl/ml retinal extract, 75 µg/ml heparin, 100 U/ml penicillin G, and 100 µg/ml streptomycin SO_4 (complete culture medium). The dishes were washed again with 10% FBS/DMEM to remove the debris after an incubation overnight (this medium was subsequently filtered through a 0.2-µm filter and was added to the cultures). The cultures were maintained at 37°C in 5% CO_2 and were provided with fresh media every 4–6 days. Some scattered pericytes were removed by weeding [Yan et al., 1996] before day 5 in culture. Early passage cells were frozen for later use. Cells from passages 3–6 were used for experiments.

For isolation of bovine retinal pericytes, the digestion with 0.3% collagenase/dispase was increased to 2–3 h, and the tissue fragments were filtered through a 40-µm nylon sieve. The filtrate (containing released cells) was collected, washed twice with DMEM containing 10% FBS, resuspended in 10% FBS/DMEM, and plated onto plastic dishes for an incubation overnight. Dishes were rinsed several times to remove debris and unattached cells; cell growth was achieved in 10% FBS in DMEM. No capillary fragments were observed in these preparations because only individual cells were attached to the dishes. After 1.5–2 weeks in culture, primary cultures of pure pericytes were apparent. Occasionally, a few smooth muscle cell colonies appeared in the pericyte cultures; these cells were removed by weeding [Yan et al., 1996]. By 2–3 weeks, confluent pericytes were harvested and frozen for later use.

Immunocytochemistry

The EC and pericyte cultures were fixed in 100% methanol for 10 min at 4°C, washed with phosphate-buffered saline (PBS), and incubated with 10% normal goat serum for 2 h. Subsequently, they were exposed to primary antibodies in a humid chamber for 2 h at room temperature: rabbit anti-human von Willebrand factor (vWF; 1:200 dilution of a 5.4 g/l-stock solution; DAKO, Carpenteria, CA), monoclonal mouse anti-α-smooth muscle actin (1:800 dilution of an 8.1-mg/ml stock solution; Sigma), polyclonal rabbit anti-human glial fibrillary acidic protein (GFAP; 1:1,000 dilution of an 8.7-mg/ml stock solution; BioMaker, Rehovot, Israel). Negative controls included replacement of primary antibodies by normal mouse IgG1 or PBS. After several washes in PBS, we added secondary antibodies conjugated with Texas Red or fluorescein isothiocyanate at a dilution of 1:200 (Sigma). Slides were mounted in 80% glycerol and were photographed with a Nikon fluorescence microscope. For the uptake of fluoresceinated, acetylated low-density lipoprotein (DiI-ac-LDL; Biomedical Technologies, Stoughton, MA), cells were incubated 4 h at 37°C in culture medium containing 5 µg/ml DiI-Ac-LDL, fixed in 2% paraformaldehyde, and mounted in glycerol prior to photography.

Cell Culture Conditions for Study of Apoptosis

Retinal microvascular EC were grown and maintained in DMEM containing 10% FBS, in the absence of retinal extract and heparin, in a humid atmosphere of 5% CO_2 at 37°C for 1–2 days prior to the experiments. Cells were detached by a rinse with Ca^{+2}/Mg^{+2}-free PBS (Sigma) followed by exposure to 0.05% trypsin-0.02% EDTA (Gibco, Grand Island, NY). Detached cells were resuspended in DMEM containing 10% FBS and antibiotics and were plated on FN-coated dishes or wells for 24–48 h (all cells were attached and spread within this interval). Subsequently, the culture medium was changed to 2% FBS/DMEM containing different concentrations of TGF-β1 (R&D Systems, Minneapolis, MN) or 2% FBS/DMEM alone for 24–48 h. Pericytes plated on plastic wells were treated identically.

DNA Synthesis Assay

Cells were palted in 24-well plates at a density of 2×10^4 EC or 1×10^4 pericytes/well in 10% FBS in DMEM and were grown for 48 h. The culture medium was changed to 2% FBS in DMEM, and TGF-β1 was added subsequently. Control cultures were grown in DMEM containing 2% FBS only. Cultures were incubated for 48 h and were exposed to 2 Ci/ml [methyl-^{3}H]-thymidine (81.3 Ci/mmol, 1 mCi/ml; New England Nuclear, Boston, MA) for the last 4 h. They were washed three times with PBS, incubated in ice-cold 10% trichloroacetic acid for 1 h, washed twice with anhydrous ethanol at −20°C, and air-dried. Three hundred microliters of NaOH (0.2N) were added to each well for 30 min at 68°C. The counts per minute (cpm) incorporated into DNA were quantified by liquid scintillation counting in 3 ml Ecolume (ICN, Irvine, CA).

Cell Viability Assays

Determination of cell viability was done by a trypan blue exclusion assay. Cells were plated and treated as described above. Attached cells were treated with trypsin, resuspended, and incubated with trypan blue dye for 2–5 min. Live cells were counted by hemacytometer.

Blocking experiments were performed on retinal EC plated on FN-coated 48-well plates in DMEM containing 10% FBS for 48 h. The medium was changed to DMEM with either 2% or 5% FBS. TGF-β1 (2 ng/ml) or TGF-β1 (2 ng/ml) + anti-TGF-β neutralizing antibody (R&D Systems; 7 μg/ml) was added simultaneously to different wells. Control wells contained DMEM with 2% or 5% FBS only. After a 24-h incubation, a cell viability assay was performed, as described above.

Double-Staining With ApopTag and Hoechst 33258 Dye

EC in 2% FBS/DMEM were incubated on FN-coated, 35-mm dishes for 24 h in the presence or absence of TGF-β1 (4 ng/ml). Cells were fixed with 2% paraformaldehyde in PBS and were rendered permeable with 0.5% Nonidet P-40 (NP-40) for 5 min. The cells were next incubated with digoxigenin-dUTP terminal dioxynucleotide transferase and were subsequently labeled with fluorescein-conjugated antibody to digoxigenin, according to the manufacture's protocol (ApopTag plus, In Situ Apoptosis Detection Kit, Oncor, Gaithersburg, MD). Morphological changes in the nuclear chromatin of cells undergoing apoptosis were also detected by a counterstain with Hoechst 33258 flurochrome (4 μg/ml in distilled water; Molecular Probes Inc., Eugene, OR) for 10 min at room temperature. The slides were mounted with 80% glycerol and were photographed under a Nikon fluorescence microscope. Detached cells in TGF-β1-treated dishes were collected, pelleted, fixed in 2% paraformaldehyde for 20 min, and washed with PBS. The cells were resuspended in PBS at 10^6 cells/100 μl, 200 μl of which was pipetted onto one of several slides. The cells were stained with ApopTag and Hoechst 33258 as described above and were photographed.

Gel Analysis of DNA Fragmentation

Cultured retinal EC were collected with a cell scraper, centrifuged (500*g*, 5 min), and washed with cold PBS. The cell pellet was resuspended in lysis buffer (10 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid [EDTA], 0.2% Triton X-100, pH 7.5). After a 30-min incubation on ice, the lysate was centrifuged at 13,000*g* for 20 min at 4°C. The supernate was collected and incubated with RNAase A (50 μg/ml) for 30 min at 37°C. DNA was purified by phenol-chloroform extraction and by precipitation at −70°C for 24 h after addition of 2 volumes of ice-cold ethanol and 1/10 volume of 3 M sodium acetate (pH 5.3). The DNA was subsequently washed with cold 70% ethanol, air-dried, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The sample was resolved by electrophoresis in a 1.5% agarose gel. DNA was visualized by staining with ethidium bromide (0.5 μg/ml), and the gel was photographed under ultraviolet light with a Polaroid camera.

Western Blot Analyses

Cells were lysed in a NP-40 buffer (0.5% NP-40, 10% glycerol, 1 mM dithiothreitol [DTT], 2.5 mM ethylene glycol bis [(β-aminoethyl ether)-tetraacetate (EGTA)], 5 mM EDTA, 150 mM NaCl, 50 mM Hepes, [pH 7.4], (1 mM NaF, and 0.5 mM Na vanadate) containing a complete protease inhibitor cocktail (Boehringer Mannheim Biochemical). Protein concentrations were determined by a Bradford assay (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as a standard. Cell lysates (20 μg protein) were solubilized in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and were heated for 5 min at 100°C with 50 mM DTT. Proteins were resolved by SDS-PAGE on 4/15% polyacrylamide minigels and were electrotransferred onto nitrocellulose membranes (0.45-μm pore size). The blots were stained with 0.1% amido black (in 10% acetic acid and 20% methanol) to verify equal loading and transfer efficiency. Blots were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20 overnight at 4°C and were probed with 1 μg/ml mouse anti-human p53 monoclonal antibody (Pharmingen, San Diego, CA) and 1 μg/ml mouse anti-human WAF1(ab-1) monoclonal antibody (Oncogene Research Product, Cambridge, MA) for 2 h at room temperature. After several washes in PBS with 0.05% Tween 20, signals were detected by horseradish peroxidase-conjugated secondary goat anti-mouse antibody at a dilution of 1:2,000 (Bio-Rad Laboratories, Richmond, CA) for 45 min at

room temperature. After extensive washes, electrochemical luminescence was developed (Amersham, Arlington Heights, IL). Blots were exposed to RP X-Omat film (Eastman Kodak, Rochester, NY). The blot was stripped with stripping buffer (100 mM β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, and 2% w/v SDS), and anti-enolase IgG (a gift from Dr. Lindsay Miles, The Scripps Institute and Research Foundation, La Jolla, CA) was used to reprobe the blot as an internal control.

RESULTS

Characterization of Cultured Retinal EC and Pericytes

The morphology and phenotypic characteristics of the retinal EC and pericytes used in this study are shown in Figure 1. Retinal EC exhibited a cobblestonelike, contact-inhibited monolayer (Fig. 1A). These cells were identified as endothelial by the uptake of DiI-ac-LDL, by reactivity with an antibody against vWF, and by the absence of labeling with anti-smooth muscle alpha-actin IgG (Fig. 1B, C, D, respectively). In contrast, pericytes exhibited an irregular morphology (Fig. 1E), characterized by a multilayered growth pattern without the "hill and valley" overgrowth typical of vascular smooth muscle cells. Moreover, these cells expressed smooth muscle alpha-actin (Fig. 1F) but neither GFAP (a marker for astrocytes) nor the EC markers noted above (data not shown).

Effect of TGF-β1 on Cell Proliferation and Viability

After approximately 20 h of culture in the presence of TGF-β1, we observed a significant number of detached retinal EC. ^{3}H-thymidine incorporation assays indicated that TGF-β1 inhibited DNA synthesis of EC in a concentration-dependent manner (Fig. 2A). We examined cell viability by trypan blue assay and found that the number of live cells was decreased significantly as a function of increasing concentration

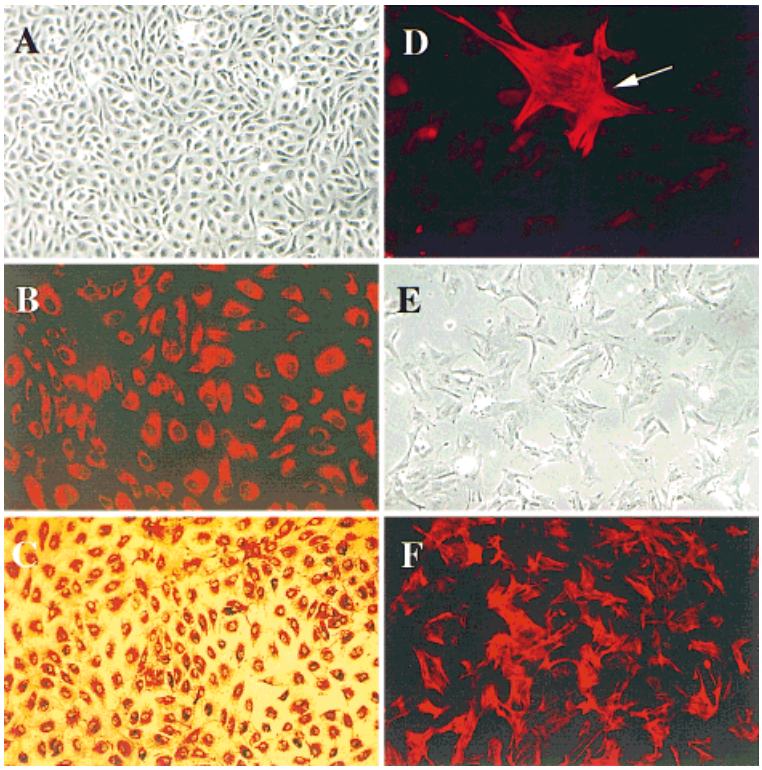

Fig. 1. Characterization of cultured retinal capillary endothelial cells (EC) and pericytes. **A:** A confluent, contact-inhibited monolayer of EC at passage 3 (phase-contrast photograph). **B:** Binding and internalization of DiI-ac-LDL by cultured EC (4 h incubation). **C:** Monolayer of EC labeled for von Willebrand factor (vWF) by anti-vWF IgG in conjunction with the avidin-biotin-peroxidase technique. **D:** Retinal EC incubated with anti-smooth muscle α-actin IgG show no reactivity, whereas pericytes exhibited smooth muscle α-actin fibers throughout the cytoplasm (arrow). **E:** Pericytes in subconfluent culture exhibit irregular morphology at passage 2 (phase-contrast photograph). **F:** Pericytes express smooth muscle α-actin IgG. Magnification ×100.

of TGF-β1 (Fig. 2B), in agreement with the data shown in Figure 2A. Decreases in both DNA synthesis and cell numbers were likely due to (1) temporary arrest of the cell cycle or (2) induction of cell death (possibly apoptosis). A TGF-β1-blocking experiment was performed first to ensure that the observed effects on EC were indeed due to TGF-β1. Retinal EC were grown for 24 h on FN-coated wells in DMEM with 10% FBS for 24 h; subsequently, the concentration of FBS was reduced to 2%, and TGF-β1 and TGF-β1 plus anti-TGF-β1 neutralizing antibody were added to different wells. As shown in Figure 3, the anti-TGF-β1 antibody abrogated cell death because cell numbers in the presence of the antibody were nearly identical to those of control cells, and their morphology was similar to that of control cells (data not shown). The experiment was also repeated in 5% FBS, with identical results (not shown).

In contrast, no detached cells were observed in pericyte cultures after the addition of TGF-β1 (up to 10 ng/ml for 2 days). Incorporation of ^{3}H-thymidine by pericytes was decreased in a concentration-dependent manner after a 48-h incubation with TGF-β1 (Fig. 4A). However, simultaneous experiments showed no significant change in cell number as a function of increasing concentration of TGF-β1 (Fig. 4B). The data indicate that DNA synthesis in pericytes was inhibited by TGF-β1, without appreciable changes in cell number over a period of 48 h. These results contrast sharply with those observed for EC. However, after prolonged periods of culture in the presence of TGF-β1 (1 week), decreases in pericyte cell number were seen (not shown).

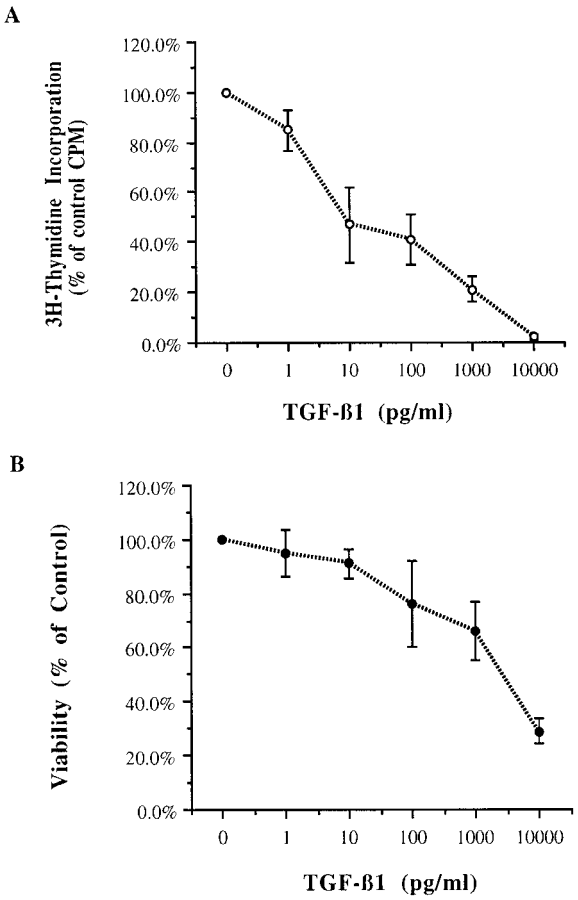

Fig. 2. Effect of TGF-β1 on retinal EC: DNA synthesis and cell proliferation. Retinal EC (2×10^4) were incubated in 10% FBS/DMEM for 48 h. Subsequently, media were changed to 2% FBS in DMEM containing TGF-β1 (0, 1, 10, 100, 1,000, or 10,000 pg/ml) for 48 h. Cells were pulsed during the last 4 h with ^{3}H-thymidine at 2 μCi/ml **(A)** or were counted as trypan blue-negative cells by hemacytometer. **(B)** Data are mean ± S.D.; values were normalized to control counts or to counts per minute incorporated by controls cultured in 2% FBS/DMEM without TGF-β1.

TGF-β1-Induced Cell Death Occurs by Apoptosis

After retinal endothelial monolayers were incubated with TGF-β1 for 20 h, many cells exhibited shrinkage, nuclear and cytoplasmic condensation, membrane blebbing, and loss of cell–cell contact. Subsequently, the cells began to break apart into small apoptotic bodies, became detached, and floated in culture dishes (Fig. 5). To observe the condensed and fragmented nuclei and to identify potentially apoptotic cells, we performed double labeling with Hoechst 33258 dye and ApopTag reagent (Fig. 6). The images show Hoechst staining on control cells (Fig. 6A), TGF-β1-treated cells (Fig. 6B), and detached cells after TGF-β1 treatment (Fig. 6C). ApopTag labeling of the same fields is shown in Figure 6a–c. Arrows in (Fig. 6B) indicate the brightly staining, fragmented nuclei that were also labeled with the ApopTag reagent (Fig. 6b). These profiles were not seen in control cells (Fig. 6A,a). Anti-TGF-β1 neutralizing antibody blocked the cell fragmentation induced by TGF-β1 (not shown). Detached floating cells collected from the cultures that had been incubated with TGF-β1 are shown in Figure 6C. All the detached cells exhibited fragmented and condensed nuclei (Fig. 6C) and were stained with ApopTag (Fig. 6c). Few intact nuclei were observed in the collected detached cells. Analysis of DNA from TGF-β1-treated EC demon-

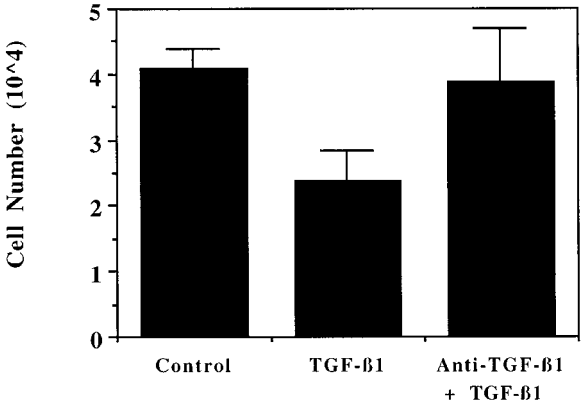

Fig. 3. Anti-TGF-β1 neutralizing antibody blocks the inhibitory effect on EC proliferation induced by TGF-β1. Retinal EC were plated in 48-well culture plates coated with fibronectin. After adherence, cells received 2% FBS/DMEM (control); TGF-β1 (2 ng/ml) in 2% FBS/DMEM; or TGF-β1 (2 ng/ml) + anti-TGF-β1 neutralizing antibody (7 μg/ml) in 2% FBS/DMEM. After 24 h, live cells were counted by hemacytometer. Data are mean ± S.D.

strated fragmentation of chromatin into nucleosomal ladders (Fig. 7). These data were indicative of apoptosis, or programmed cell death, in retinal EC.

Interestingly, not all of the retinal EC were sensitive to TGF-β1-induced apoptosis. We removed the detached apoptotic cells and the apoptotic cells that were rounded but still attached to the substrate. The remaining attached EC were incubated with Hoechst 33258 dye and ApopTag reagent. Almost all the remaining cells showed intact nuclei and were not labeled by ApopTag. Thus, this population appeared to be resistant to TGF-β1-mediated apoptosis (Fig. 5C). Moreover, these cells exhibited a change in morphology after exposure to TGF-β1 in the presence of 2% FBS. Despite a more elongate shape in comparison with the shape of untreated retinal EC, they lacked smooth muscle α-actin and exhibited uptake of DiI-ac-LDL (data not shown).

Although some retinal EC with apoptotic morphology were found in control cultures (likely a consequence of the decrease in FBS from 10% to 2%), the number was insignificant when compared with that seen in cultures treated with TGF-β1.

Expression of p53 and p21$^{waf1/cip1}$ Proteins in EC and Pericytes

To determine whether the apoptosis induced by TGF-β1 was associated with changes in the

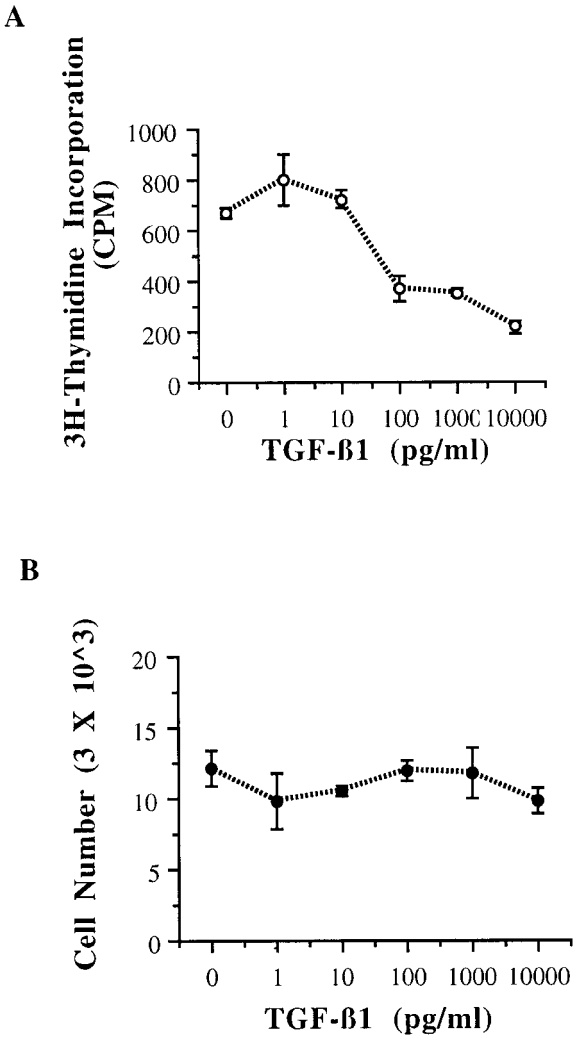

Fig. 4. Effect of TGF-β1 on DNA synthesis and cell proliferation in retinal pericytes. Retinal pericytes (1×10^4) were incubated in 10% FBS/DMEM for 24 h. Subsequently, media were changed to 2% FBS in DMEM containing TGF-β1 (0, 1, 10, 100, 1,000, or 10,000 pg/ml). After an incubation of 48 h, cells were pulsed for the last 4 h with ^{3}H-thymidine at 2 μCi/ml **(A)** or were counted by hemacytometer **(B).** Data are mean ± S.D.

expression of p53 and/or p21$^{waf1/cip1}$, we made cell extracts from either control or TGF-β1-treated EC. Detached cells (TGF-β1 apoptosis-sensitive cells) and attached cells (TGF-β1 apoptosis-resistant cells) from TGF-β1-treated cultures were analyzed separately. Monoclonal antibodies against p53 and p21$^{waf1/cip1}$ were used simultaneously on the same nitrocellulose blot. The apoptotic EC exhibited increased expression of p53 protein when compared with that of attached cells and/or untreated control cells (160% over control) (Fig. 8A). Attached cells contained similar levels of p53 in comparison

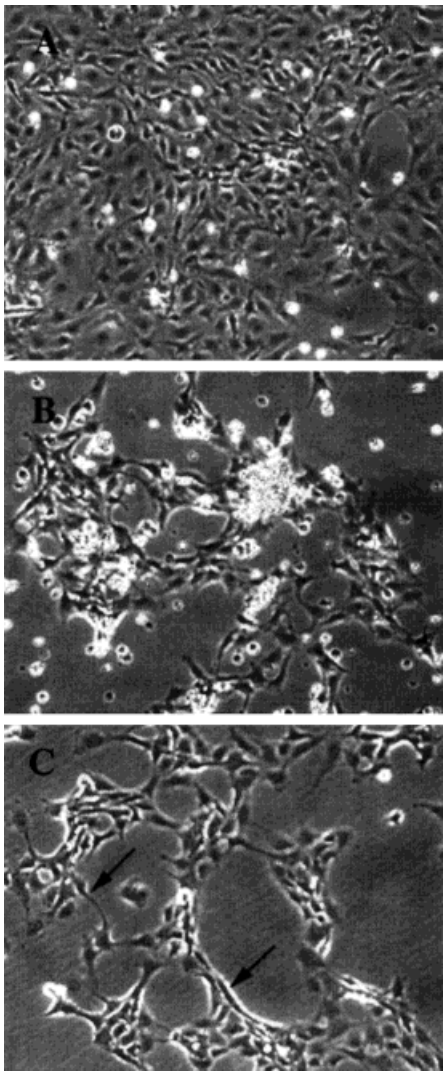

Fig. 5. Changes in morphology of retinal EC after treatment with TGF-β1. **A:** EC in 2% FBS/DMEM for 24 h. **B:** Cells treated for 24 h with 4 ng/ml TGF-β1 in 2% FBS/DMEM. **C:** TGF-β1 nonapoptotic EC: cells were treated for 24 h with 4 ng/ml TGF-β1 in 2% FBS/DMEM. Detached cells and cells that were rounded but partly attached were removed by several washes. Arrows indicate elongated cells that have lost their typical cobblestone morphology. Magnification ×110.

with those of the control cells. Interestingly, $p21^{waf1/cip1}$ was decreased significantly in apoptotic detached cells in comparison with control cells and/or attached cells (5.5% of control). However, the attached cells expressed essentially the same amount of $p21^{waf1/cip1}$ as control cells. Staining with amido black (not shown) and immunoblotting with anti-enolase IgG (Fig. 8A) confirmed nearly equal loading of cell-extract protein (20 µg/lane).

The same experiments were performed with pericytes (Fig. 8B). The p53 and $p21^{waf1/cip1}$ proteins remained relatively unchanged between the control (untreated) and TGF-β1 (2 and 7 ng/ml, respectively)-treated samples. The protein $p21^{waf1/cip1}$ was barely detectable and p53 was relatively low in comparison with the level shown in EC. Probing with anti-enolase IgG showed nearly equal loading of all the samples (20 µg/lane).

DISCUSSION

Retinal microvascular EC are unique among vascular endothelia [Schor and Schor, 1986; Thieme et al., 1995], because they are subject to regulation by (a) the various cytokines, extracellular matrix components, and matricellular proteins produced by retinal cells (including neurons) [Tanihara et al., 1997; Yan et al., 1998]; (b) interactions with pericytes, astrocytes, and Muller cells [Jiang et al., 1995; Puro, 1995]; and (c) local changes in oxygen [Chan-Ling et al., 1995]. Although the propagation of pure cultures of retinal micovascular EC in vitro has been difficult, these cells, isolated successfully from several species, have been studied with respect to their biochemical properties, interaction with cells such as pericytes and astrocytes, and responses to exogenous factors and matrix proteins, all of which contribute to the process of retinal neovascularization.

In the present report, we have demonstrated that TGF-β1 induces apoptotic cell death in cultured retinal EC, but not in retinal pericytes. Apoptosis is obvious in retinal EC after 20 h of incubation with exogenous TGF-β1 as a function of concentration. Because serum deprivation induces endothelial apoptosis [Araki et al., 1990a,b], we attempted to minimize this effect by performing our experiments in 2% FBS, in which both retinal EC and pericytes are viable. The extent of cell death in 2% versus 10% FBS was insignificant in comparison with that observed in the TGF-β1-treated cells. We also found that the degree of apoptosis induced by TGF-β1 was slightly lower in 5% versus 2% FBS. This result indicates that the apoptosis we described is not due to serum deprivation, although serum may in part compensate for some of the apoptotic effects induced by TGF-β1.

Our data also show that TGF-β1 inhibited ^{3}H-thymidine incorporation in both retinal EC and pericytes. The mechanism of TGF-β-induced antiproliferation is not fully understood, but for many cell types it is clear that TGF-β1 causes growth arrest in the G1 phase of the cell

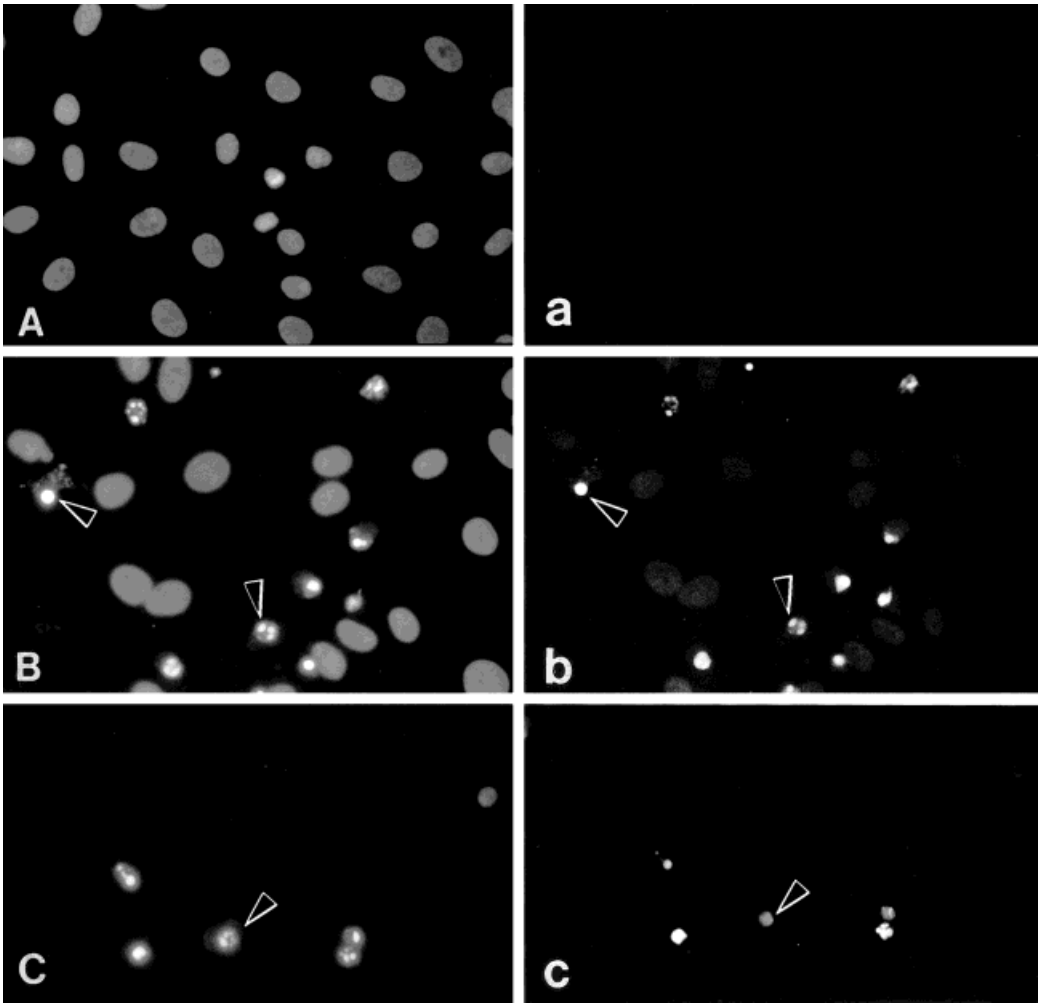

Fig. 6. TGF-β1 induces DNA fragmentation in retinal EC. **A:** Control EC in 2% FBS/DMEM for 24 h. **B:** EC treated with TGF-β1 (4 ng/ml) in 2% FBS/DMEM for 24 h. **C:** Detached cells (B) were collected, pelleted, and spread on slides. DNA was stained with Hoechst 33258 fluorochrome (A–C). The fluorescence micrographs show condensed and fragmented apoptotic cell nuclei (arrows in B and C). **a–c:** ApopTag staining of the same fields shown in A–C. Arrows in b and c indicate apoptotic cells that correspond to the fragmented DNA detected by the Hoechst 33258 dye in B and C. Magnification ×210.

cycle [Massague, 1990; Sporn and Roberts, 1992]. This arrest is due in part to the regulation of G1 cyclins/cyclin-dependent kinases by induction of their inhibitors $p21^{waf1/cip1}$, $p27^{Kip1}$, p16, and $p15^{INK4B}$, and by the reduction of c-myc [Ewen et al., 1993; Geng and Weinberg, 1993; Koff et al., 1993]. Another target gene for the growth-inhibitory activity of TGF-β1 is p53 [Suzuki et al., 1992; Raynal et al., 1994; Landesman et al., 1997]. We investigated whether p53 and $p21^{waf1/cip1}$ were involved in the regulation of retinal endothelial apoptosis for the following reasons: (1) both proteins are involved in growth inhibition; (2) p53 mediates some, but not all, forms of apoptosis [Clarke et al., 1994; Strasser et al., 1994]; (3) some forms of p53-induced apoptosis are associated with increased expression of $p21^{waf1/cip1}$ [El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993]; and (4) $p21^{waf1/cip1}$ can block apoptosis during cell differentiation [Shi et al., 1994; Wang and Walsh, 1996].

Retinal EC were not uniformly sensitive to TGF-β1-mediated apoptotic cell death. In the presence of 2 ng/ml TGF-β1 in 2% FBS/DMEM, approximately 30% of the cells were apoptotic. The TGF-β1-sensitive cells (detached) exhibited significantly reduced levels of $p21^{waf1/cip1}$; in contrast, elevated levels of $p21^{waf1/cip1}$ were found in the TGF-β1-resistant cells (attached) in com-

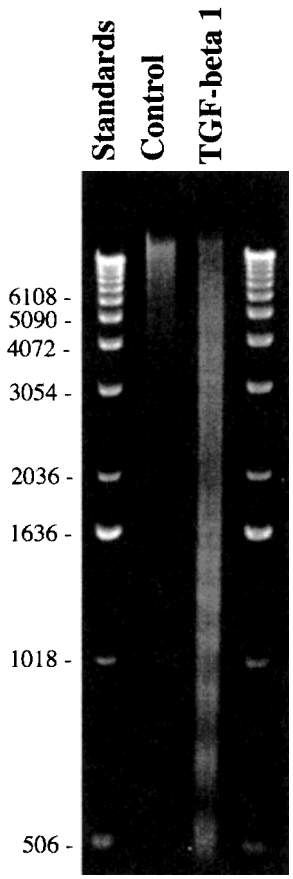

Fig. 7. Electrophoresis of DNA from control and TGF-β1-treated retinal EC. Retinal EC were incubated in 4 ng/ml of TGF-β1 for 24 h or in 2% FBS/DMEM alone (control) for 24 h. DNA was extracted from treated cells and was resolved by electrophoresis on a 1.5% agarose gel. DNA bands were visualized by staining with ethidium bromide. **Lane 1:** DNA standards (bp). **Lane 2:** Control cells, DNA extract. **Lane 3:** TGF-β1-treated cells, DNA extract.

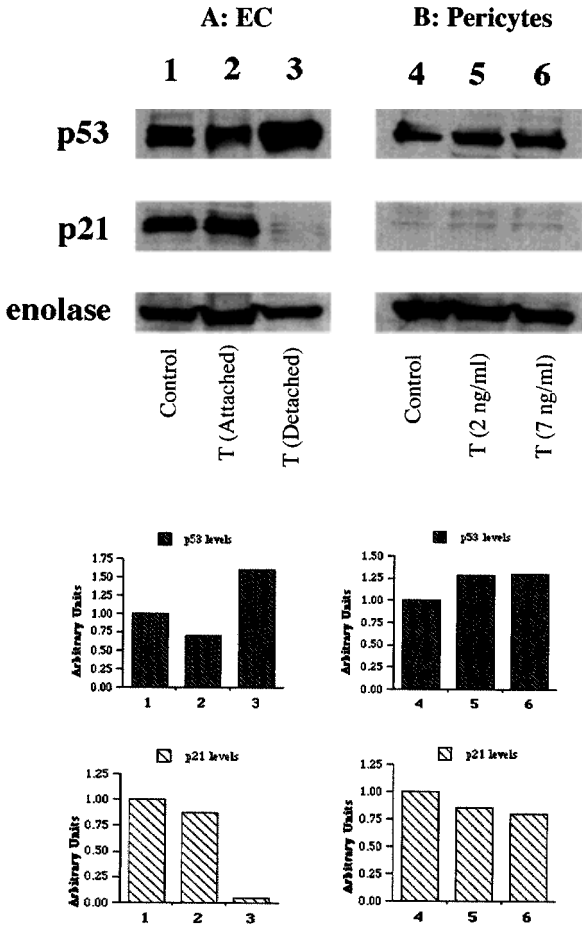

Fig. 8. Expression of $p21^{waf1/cip1}$ and p53 by retinal EC and pericytes in response to TGF-β1. Twenty micrograms of protein per lane, extracted from EC or pericytes 24 or 48 h after addition of TGF-β1, were resolved by SDS-PAGE. Immunoblotting was performed subsequently with anti-WAF1 (p21) and anti-p53 antibodies, followed by appropriate secondary antibodies. The blot was stripped and reprobed with an anti-enolase antibody as an internal control. **A:** EC. **Lane 1:** Control (2% FBS/DMEM). **Lane 2:** TGF-β1 (2 ng/ml, attached cells). **Lane 3:** TGF-β1 (2 ng/ml, detached cells). **B:** Pericytes. **Lane 4:** Control (2% FBS/DMEM, 48 h). **Lane 5:** TGF-β1 (2 ng/ml, 48 h). **Lane 6:** TGF-β1 (7 ng/ml, 48 h). T, TGF-β1. Histogram below A and B shows results of scanning densitometry of p53 and $p21^{waf1/cip1}$. The data shown are representative of three experiments. Data were normalized to the internal loading control (enolase) and were plotted relative to p53 and $p21^{waf1/cip1}$ levels in control (untreated) samples.

parison with the levels found in the detached cells (Fig. 8A). Thus, induction of $p21^{waf1/cip1}$ appears to be correlated with a resistance to TGF-β1-mediated apoptosis in retinal EC. It is also possible that, in the presence of TGF-β1, $p21^{waf1/cip1}$ protects EC that are growth-arrested from cell death. In fact, this hypothesis appears to be valid for myocyte differentiation in low serum [Wang and Walsh, 1996], and similar observations have been reported in other cell types in vitro [Shi et al., 1994; Poluha et al., 1996]. The change in morphology that we observed in the apoptosis-resistant EC cultured in TGF-β1 was in fact reminiscent of studies by Arciniegas et al. [1992], who found that TGF-β1 promoted the differentiation of EC into smooth muscle-like cells in vitro.

The p53 protein is a sequence-specific DNA-binding protein that acts as a transcription factor, and the gene encoding $p21^{waf1/cip1}$ contains a p53-responsive element. Exposure of cells to ionizing radiation or to DNA-damaging agents results in the accumulation of high levels of p53 protein and in the subsequent stimulation of $p21^{waf1/cip1}$ and cell cycle arrest, presumably for repair of the damaged DNA or for

apoptosis. In contrast, TGF-β1 can effect rapid transcriptional induction of $p21^{waf1/cip1}$ through a p53-independent pathway [Datto et al., 1995]. Nevertheless, p53 does appear to mediate growth inhibition induced by TGF-β1 [Reiss et al., 1993; Mogi et al., 1994; Raynal et al., 1994; Ewen et al., 1995], e.g., TGF-β1 controls the intracellular localization in addition to the phosphorylation pattern and the stability of p53 protein [Suzuki et al., 1992; Landesman et al., 1997]. Thus, both p53 and $p21^{waf1/cip1}$ participate in one or more pathways that transduce signals via TGF-β1 receptors. It has been shown that proliferating cells that subsequently became apoptotic exhibited an induction of p53 followed by an increase in $p21^{waf1/cip1}$ [Symonds et al., 1994; Boudreau et al., 1996; Stromblad et al., 1996; Gansauge et al., 1997]. The induction of apoptosis could result from concomitant but conflicting signals, such as stimulation of DNA synthesis and growth arrest [Stromblad et al., 1996]. In our experimental system, however, the retinal cells are for the most part growth-inhibited, and the accumulation of p53 was not associated with activation of $p21^{waf1/cip1}$ in cells undergoing TGF-β1-mediated apoptosis. Therefore, p53 may regulate apoptosis in these cells through a stimulation of bax and a diminution of the bcl-2 pathway because p53 is a transcriptional repressor of bcl-2 and an activator of bax [Miyashita et al., 1994a,b]. Alternatively, p53 may affect regulators other than $p21^{waf1/cip1}$, bcl-2, or bax. Because there are no anti-bcl-2 antibodies that crossreact with the bovine protein, we were unable to determine the bcl-2:bax ratio in retinal cells exhibiting elevated levels of p53.

Other functions of TGF-β1 may also contribute to the apoptotic response that we observed. For example, interactions between cells and extracellular matrix proteins are critical for cell survival [Meredith et al., 1993; Frisch and Francis, 1994; Boudreau et al., 1995], and TGF-β1 regulates deposition and degradation of matrix proteins and integrin expression on EC [Lyons and Moses, 1990; Massague, 1990]. Therefore, detachment of retinal EC may be due in part to changes in extracellular matrix mediated by TGF-β1. The distribution of TGF-β1 receptors on retinal capillary EC should also be considered. Expression of TGF-β receptors (type I, type II, and betaglycan) is constitutive in most cell types [Massague, 1992], and types I and II have been identified as signaling receptors for TGF-β1. A recent report has indicated that the type II receptor mediates the antiproliferative effect, whereas the type I receptor is responsible for the matrix-biosynthetic response of EC to TGF-β1 [Sankar et al., 1996]. Determination of both the levels and types of TGF-β1 receptors on retinal EC will provide valuable insight into the apoptotic effect described in the present report.

One of the unique features of retinal capillaries is the unusually high ratio (1:1) of pericytes to EC [Kohner et al., 1991]. Both their proximity and interactive capabilities predict functional, intercellular regulation. When we investigated the proliferative response of pericytes to TGF-β1, we found that proliferation was inhibited by TGF-β1 but that pericytes were resistant to TGF-β1-mediated apoptosis. This result may be due in part to the lack of response of the p53 and $p21^{waf1/cip1}$ genes to TGF-β1 in pericytes. Furthermore, p53 and $p21^{waf1/cip1}$ do not appear to be critical for the inhibition of pericyte cell growth by TGF-β1.

In the present study, we have shown that retinal microvascular EC undergo a programmed cell death in response to TGF-β1. The mechanisms underlying this phenomenon likely involve multiple pathways, one of which is associated with decreased levels of the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$. The induction of retinal endothelial apoptosis by TGF-β1 could lead to the regression of retinal neovascular capillaries and could therefore form the basis for potential therapeutic strategies for neovascular retinopathy.

ACKNOWLEDGMENTS

We thank Drs. Yi Li, Aleksandar Francki, and Kouros Motamed for their assistance and helpful discussions. We are indebted to Preston Van Hooser for providing bovine eye tissue. We also thank Dr. Anita Hendrickson for her continued interest and support of this project and for a critical reading of the manuscript. Anti-enolase IgG antibody was kindly provided by Dr. Lindsay Miles. Q.Y. was supported by grants PHS NRSA T32 GM07270 from NIGMS and T32 EY07031 from NEI.

REFERENCES

Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E (1995): Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nature Med 1:1024–1028.

Anderson DH, Guerin CJ, Hageman GS, Pfeffer BA, Flanders KC (1995): Distribution of transforming growth factor-beta isoforms in the mammalian retina. J Neurosci Res 42:63–79.

Antonelli-Orlidge A, Saunders KB, Smith SR, D'Amore PA (1989): An activated form of TGF-β is produced by cocultures of endothelial cells and pericytes. Proc Natl Acad Sci USA 86:4544–4548.

Arciniegas E, Sutton AB, Allen TD, Schor AM (1992): Transforming growth factor beta 1 promotes the differentiation of endothelial cells into smooth muscle-like cells in vitro. J Cell Sci 103:521–529.

Araki S, Shimada Y, Kaji K, Hayashi H (1990a): Apoptosis of vascular endothelial cells by fibroblast growth factor deprivation. Biochem Biophys Res Commun 168:1194–1200.

Araki S, Simada Y, Kaji K, Hayashi H (1990b): Role of protein kinase C in the inhibition by fibroblast growth factor of apoptosis in serum-depleted endothelial cells. Biochem Biophys Res Commun 172:1081–1085.

Boudreau N, Sympson CJ, Werb Z, Bissell MJ (1995): Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. Science 267:891–893.

Boudreau N, Werb Z, Bissell MJ (1996): Suppression of apoptosis by basement membrane requires three-dimensional tissue organization and withdrawal from the cell cycle. Proc Natl Acad Sci USA 93:3509–3513.

Chan-Ling T, Gock B, Stone J (1995): The effect of oxygen on vasoformative cell division. Invest Ophthalmol Vis Sci 36:1201–1214.

Choi ME, Ballermann BJ (1995): Inhibition of capillary morphogenesis and associated apoptosis by dominant negative mutant transforming growth factor-beta receptors. J Biol Chem 270:21144–21150.

Clarke AR, Gledhill S, Hooper ML, Bird CC, Wyllie AH (1994): p53 dependence of early apoptotic and proliferative responses within the mouse intestinal epithelium following gamma-irradiation. Oncogene 9:1767–1773.

D'Amore PA (1994): Mechanisms of retinal and choroidal neovascularization. Invest Ophthalmol Vis Sci 35:3974–3979.

Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang XF (1995): Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc Natl Acad Sci USA 92:5545–5549.

Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ (1995): Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. Development 121:1845–1854.

El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B (1993): WAF1, a potential mediator of p53 tumor suppression. Cell 75:817–825.

Esser P, Heimann K, Bartz-Schmidt K, Fontana A, Schraermeyer U, Thumann G, Weller M (1997): Apoptosis in proliferative vitreoretinal disorders: Possible involvement of TGF-β-induced RPE cell apoptosis. Exp Eye Res 65:365–378.

Ewen ME, Sluss HK, Whitehouse LL, Livingston DM (1993): TGF beta inhibition of Cdk4 synthesis is linked to cell cycle arrest. Cell 74:1009–1020.

Ewen ME, Oliver CJ, Sluss HK, Miller SJ, Peeper DS (1995): p53-dependent repression of CDK4 translation in TGF-beta-induced G1 cell-cycle arrest. Genes Dev 9:204–217.

Fabregat I, Sánchez A, Alvarez AM, Nakamura T, Benito M (1996): Epidermal growth factor, but not hepatocyte growth factor, suppresses the apoptosis induced by transforming growth factor-beta in fetal hepatocytes in primary culture. FEBS Lett 384:14–18.

Frisch SM, Francis H (1994): Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 124: 619–626.

Gansauge S, Gansauge F, Gause H, Poch B, Schoenberg MH, Beger HG (1997): The induction of apoptosis in proliferating human fibroblasts by oxygen radicals is associated with a p53- and p21WAF1CIP1 induction. FEBS Lett 404:6–10.

Gaudric A, Glacet BA, Clement G, Falquerho L, Barritault D, Coscas G (1990): Transforming growth factor beta in the vitreous of patients with epiretinal proliferation. Ophthalmologie 4:51–52.

Geng Y, Weinberg RA (1993): Transforming growth factor beta effects on expression of G1 cyclins and cyclin-dependent protein kinases. Proc Natl Acad Sci USA 90: 10315–10319.

Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ (1993): The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75:805–816.

Jiang B, Bezhadian MA, Caldwell RB (1995): Astrocytes modulate retinal vasculogenesis: effects on endothelial cell differentiation. Glia 15:1–10.

Kingsley DW (1994): The TGF-β superfamily: New members, new receptors, and new genetic tests of function in different organisms. Genes Dev 8:133–146.

Koff A, Ohtsuki M, Polyak K, Roberts JM, Massague J (1993): Negative regulation of G1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGF-beta. Science 260:536–539.

Kohner EM, Porta M, Hyer SL (1991): The pathogenesis of diabetic retinopathy and cataract. In Pickup J, Williams G (eds): "Textbook of Diabetes." Oxford: Blackwell, pp 564–574.

Ilio KY, Sensibar JA, Lee C (1995): Effect of TGF-beta 1, TGF-alpha, and EGF on cell proliferation and cell death in rat ventral prostatic epithelial cells in culture. J Androl 16:482–490.

Landesman Y, Bringold F, Milne DD, Meek DW (1997): Modifications of p53 protein and accumulation of p21 and gadd45 mRNA in TGF-beta 1 growth inhibited cells. Cell Signal 9:291–298.

Landstrom M, Eklov S, Colosetti P, Nilsson S, Damber JE, Bergh A, Funa K (1996): Estrogen induces apoptosis in a rat prostatic adenocarcinoma: Association with an increased expression of TGF-beta 1 and its type-I and type-II receptors. Int J Cancer 67:573–579.

Lomo J, Blomhoff HK, Beiske K, Stokke T, Smeland EB (1995): TGF-beta 1 and cyclic AMP promote apoptosis in resting human B lymphocytes. J Immunol 154:1634–1643.

Lutty GA, Merges C, Threlkeld AB, Crone S, McLeod DS (1993): Heterogeneity in localization of isoforms of TGF-β in human retina, vitreous, and choroid. Invest Ophthalmol Vis Sci 34:477–487.

Lyons RM, Moses HL (1990): Transforming growth factor β and the regulation of cell proliferation. Eur J Biochem 187:467–473.

Madri JA, Pratt BM, Tucker AM (1988): Phenotypic modulation of endothelial cells by transforming growth factor-beta depends upon the composition and organization of the extracellular matrix. J Cell Biol 106:1375–1384.

Mandriota SJ, Pepper MS (1997): Vascular endothelial growth factor-induced in vitro angiogenesis and plasminogen activator expression are dependent on endogenous basic fibroblast growth factor. J Cell Sci 110:2293–2302.

Massague J (1990): The transforming growth factor-β family. Annu Rev Cell Biol 6:597–641.

Massague J (1992): Receptors for the TGF-beta family. Cell 69:1067–1070.

Meredith JE Jr, Fazeli B, Schwartz MA (1993): The extracellular matrix as a cell survival factor. Mol Biol Cell 4:953–961.

Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B, Reed JC (1994a): Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene 9:1799–1805.

Miyashita T, Harigai M, Hanada M, Reed JC (1994b) Identification of a p53-dependent negative response element in the bcl-2 gene. Cancer Res 54:3131–3135.

Mogi Y, Kato J, Horimoto M, Takimoto R, Murakami T, Hirayama A, Kohgo Y, Watanabe N, Niitsu Y (1994): Close correlation between the dephosphorylation of p53 and growth suppression by transforming growth factor-beta 1 in nasopharyngeal carcinoma cells transduced with adenovirus early region genes. Jpn J Cancer Res 85:459–463.

Montesano R, Vassalli JD, Baird A, Guillemin R, Orci L (1986): Basic fibroblast growth factor induces angiogenesis in vitro. Proc Natl Acad Sci USA 83:7297–7301.

Ohta S, Yanagihara K, Nagata K (1997): Mechanism of apoptotic cell death of human gastric carcinoma cells mediated by transforming growth factor β. Biochem J 324:777–782.

Orlidge A, D'Amore PA (1987): Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. J Cell Biol 105:1455–1462.

Pepper MS (1997): Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. Cytokine Growth Factor Rev 8:21–43.

Phillips GD, Whitehead RA, Stone AM, Ruebel MW, Goodkin ML, Knighton DR (1993): Transforming growth factor beta (TGF-B) stimulation of angiogenesis: An electron microscopic study. J Submicrosc Cytol Pathol 25:149–155.

Pierce DF Jr, Gorska AE, Chytil A, Meise KS, Page DL, Coffey RJ Jr, Moses HL (1995): Mammary tumor suppression by transforming growth factor beta 1 transgene expression. Proc Natl Acad Sci USA 92:4254–4258.

Poluha W, Poluha DK, Chang B, Crosbie NE, Schonhoff CM, Kilpatrick DL, Ross AH (1996): The cyclin-dependent kinase inhibitor p21 (WAF1) is required for survival of differentiating neuroblastoma cells. Mol Cell Biol 16: 1335–1341.

Puro DG (1995): Growth factors and Muller cells. Prog Eye Retinal Res 15:89–101.

Raynal S, Jullien P, Lawrence DA (1994): Transforming growth factor-beta 1 enhances serum-induced dephosphorylation of the P53 protein in cell lines growth-inhibited by this factor. Growth Factors 11:197–203.

Reiss M, Vellucci VF, Zhou ZL (1993): Mutant p53 tumor suppressor gene causes resistance to transforming growth factor beta 1 in murine keratinocytes. Cancer Res 53:899–904.

Roberts AB, Sporn MB (1989): Regulation of endothelial cell growth, architecture, and matrix synthesis by TGF-β1. Am Rev Respir Dis 140:1126–1128.

Rotello RJ, Lieberman RC, Purchio AF, Gerschenson LE (1991): Coordinated regulation of apoptosis and cell proliferation by transforming growth factor beta 1 in cultured uterine epithelial cells. Proc Natl Acad Sci USA 88:3412–3415.

Sato Y, Rifkin DB (1989): Inhibition of endothelial cell movement by pericytes and smooth muscle cells: Activation of a latent transforming growth factor-beta 1-like molecule by plasmin during co-culture. J Cell Biol 109: 309–315.

Sankar S, Mahooti-Brooks N, Bensen L, McCarthy TL, Centrella M, Madri JA (1996): Modulation of transforming growth factor β receptor levels on microvascular endothelial cells during in vitro angiogenesis. J Clin Invest 97:1436–1446.

Schor AM, Schor SL (1986): The isolation and culture of endothelial cells and pericytes from the bovine retinal microvasculature: A comparative study with large vessel vascular cells. Microvasc Res 32:21–38.

Selvakumaran M, Reed JC, Liebermann D, Hoffman B (1994): Progression of the myeloid differentiation program is dominant to transforming growth factor-beta 1-induced apoptosis in M1 myeloid leukemic cells. Blood 84:1036–1042.

Shepro D, Morel NML (1993): Pericyte physiology. FASEB J 7:1031–1038.

Shi L, Nishioka WK, Th'ng J, Bradbury EM, Litchfield DW, Greenberg AH (1994): Premature p34cdc2 activation required for apoptosis. Science 263:1143–1145.

Sporn MB, Roberts AB (1992): Transforming growth factor-beta: recent progress and new challenges. J Cell Biol 119:1017–1021.

Stone J, Maslim J (1997): Mechanisms of retinal angiogenesis. Prog Eye Retinal Res 16:157–181.

Strasser A, Harris AW, Jacks T, Cory S (1994): DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. Cell 79:329–339.

Stromblad S, Becker JC, Yebra M, Brooks PC, Cheresh DA (1996): Suppression of p53 activity and p21WAF1/CIP1 expression by vascular cell integrin alpha Vbeta3 during angiogenesis. J Clin Invest 98:426–433.

Suzuki K, Ono T, Takahashi K (1992): Inhibition of DNA synthesis by TGF-beta 1 coincides with inhibition of phosphorylation and cytoplasmic translocation of p53 protein. Biochem Biophys Res Commun 183:1175–1183.

Symonds H, Krall L, Remington L, Saenz-Robles M, Lowe S, Jacks T, Van-Dyke T (1994): p53-Dependent apoptosis suppresses tumor growth and progression in vivo. Cell 78:703–711.

Tanihara H, Inatani M, Honda Y (1997): Growth factors and their receptors in the retina and pigment epithelium. Prog Retina Eye Res 16:271–301.

Thieme H, Aiello LP, Takagi H, Ferrara N, King G (1995): Comparative analysis of vascular endothelial growth factor receptors on retinal and aortic vascular endothelial cells. Diabetes 44:98–103.

Tsukada T, Eguchi K, Migita K, Kawabe Y, Kawakami A, Matsuoka N, Takashima H, Mizokami A, Nagataki S (1995): Transforming growth factor beta 1 induces apoptotic cell death in cultured human umbilical vein endothelial cells with down-regulated expression of bcl-2. Biochem Biophys Res Commun 210: 1076–1082.

Wang J, Walsh K (1996): Resistance to apoptosis conferred by Cdk inhibitors during myocyte differentiation. Science 273:359–361.

Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D (1993): p21 is a universal inhibitor of cyclin kinases. Nature 366:701–704.

Yamamoto M, Maehara Y, Sakaguchi Y, Kusumoto T, Ichiyoshi Y, Sugimachi K (1996): Transforming growth factor-beta 1 induces apoptosis in gastric cancer cells through a p53-independent pathway. Cancer 77:1628–1633.

Yan Q, Vernon RB, Hendrickson AE, Sage EH (1996): Primary culture and characterization of microvascular endothelial cells from Macaca monkey retina. Invest Ophthalmol Vis Sci 37:2185–2194.

Yan Q, Sage EH, Hendrickson AE (1998): SPARC is expressed by ganglion cells and astrocytes in bovine retina. J Histochem Cytochem 46:3–10.