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Extracellular Matrix Inhibits Apoptosis and Enhances Endothelial Cell Differentiation by a NFκB-Dependent Mechanism

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Abstract Hormonal and environmental factors that control the growth, differentiation, and regression of the vasculature are of fundamental importance in tumorigenesis and in the choice of therapeutic strategies. To test the hypothesis that estradiol (E_2) and basement membrane proteins would affect the survival of vascular endothelial cells (EC), immortalized human umbilical vein endothelial cells (ECV304) were examined for their response to the chemotherapeutic drugs taxol and etoposide. ECV cell apoptosis was inhibited by E_2 (taxol only) or attachment to extracellular matrix (ECM) (taxol or etoposide). E_2 increased ECV growth, while ECM binding resulted in growth arrest and differentiation. Apoptosis was associated with decreased levels of Bcl-2 and p21 proteins. E_2 prevented down-regulation of p21 and Bcl-2 induced by taxol but did not prevent the down-regulation of p21 induced by etoposide, consistent with the failure of E_2 to inhibit etoposide-induced cell death. However, ECM prevented p21 and Bcl-2 down-regulation induced by taxol or etoposide. Persistent activation of NFκB occurred after attachment of ECV cells to ECM, suggesting a role in survival or differentiation. IκBα levels were not affected by taxol but were reduced by etoposide treatment, while IκBβ levels did not change with drug treatment. E_2 did not alter the levels of IκBα or IκBβ. Interestingly, levels of IκBα and IκBβ declined in etoposide-treated ECV cells on ECM concomitant with the elevation of NFκB, suggesting that in these cells degradation of IκB may be responsible for NFκB activation. In agreement with these data, anti-sense NFκB treatment of ECV cells inhibited differentiation on ECM, but did not affect cell survival. In conclusion, culture of ECV cells on ECM or treatment with E_2 inhibited apoptosis. NFκB activation by ECM was necessary for cellular differentiation, rather than inhibition, of apoptosis. J. Cell. Biochem. 73:321–331, 1999. Published 1999 Wiley-Liss, Inc.†

Key words: apoptosis; endothelial cells; extracellular matrix; nuclear factor; estradiol

Endothelial cell (EC) growth, differentiation, and apoptosis are regulated events during angiogenesis [Risau, 1997]. The similarities between tumor cell growth and invasion and the formation of neovessels in tumors have led to the development of strategies to target both endothelial and tumor cell proliferation and invasion for therapeutic purposes [Liotta et al., 1991]. Many chemotherapeutic drugs are most effective against rapidly growing tumor cells and would therefore be expected to inhibit rapidly growing EC [Bhalla and Harris, 1998]. However, EC in quiescent and stable vessels in established tumors (which are often present at diagnosis) may not be as responsive to agents that target neovessels. For these reasons, it is important to determine how EC respond to chemotherapeutic drugs under conditions associated with cell proliferation or growth arrest.

Most primary and some immortalized EC have been shown to retain hormonal/growth factor activities in culture and to respond to their extracellular environment by initiating cellular differentiation [Montesano, 1992]. Cell differentiation on ECM proteins such as collagen [Montesano et al., 1986], fibrin [Chalupowicz et al., 1995], or EHS matrix, Matrigel™ [Kubota et al., 1988] is often associated with

Abbreviations used: E2, 17β-estradiol; EC, endothelial cell; ECM, extracellular matrix; ECV304, immortal human umbilical vein endothelial cell; Huvec, human umbilical vein endothelial cell; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum.

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cell cycle arrest, activation of proteases, and invasion/remodeling of ECM. In addition, EC express abundant estrogen receptors, and estradiol can enhance EC growth [Venkov et al., 1996], prevent apoptosis [Spyridopoulos et al., 1997], and support differentiation [Morales et al., 1995]. How these diverse effects of estradiol are mediated in neovessels in vivo is unclear. However, several recent studies have suggested that regulation of NFκB activity may be important in EC survival [Karsan, 1998; Van Antwerp et al., 1998; Beg and Baltimore, 1996] and differentiation [Shono et al., 1996].

We have shown previously that human umbilical vein endothelial cell (Huvec) apoptosis occurs after the withdrawal of fibroblast growth factors (FGF) or treatment with tumor necrosis factor-α (TNF-α) [Yang et al., 1996]. Apoptosis was characterized by cellular protein Tyr-dephosphorylation, reduced FAK-P, loss of focal contacts, actin depolymerization, and DNA laddering. Huvec survival and differentiation were enhanced by attachment to ECM and were associated with activation of MAPK and expression of p21. To further investigate the intracellular signaling events involved in endothelial cell death and differentiation, an immortalized Huvec cell (ECV) was used. ECV cells have been shown to respond to extracellular growth factors, are contact inhibited, and differentiate when grown on EHS matrix [Takahashi et al., 1990]. In the present studies, we found ECV cells to be resistant to TNF-α-induced apoptosis, but treatment with the chemotherapeutic drugs taxol or etoposide resulted in cell death. To investigate the regulation of cell growth and apoptosis in these cells, the MTT dye conversion assay, cell cycle analysis (fluorescence-activated cell sorting [FACS]), and DNA laddering were used. Expression of proteins involved in cell survival such as Bcl-2, p21, estradiol receptor (ER), and NFκB were assessed with specific antibody immunoblotting and electrophoretic mobility shift assays (EMSA). The levels of proteins that regulate NFκB activity, IκBα and IκBβ, were measured using immunoblot procedures. We found that ECV cells regulate the expression of several genes that control cell growth, apoptosis, and differentiation and are therefore a good model with which to study the response of endothelial cells to apoptotic stimuli.

MATERIALS AND METHODS

Reagents

Taxol, etoposide, and 17β-estradiol were obtained from Sigma Chemical Co. (St. Louis, MO). The $ICI_{182,780}$ compound was a generous gift from Dr. A.E. Wakeling (Zeneca Pharmaceuticals, Cheshire, England). Trypsin/EDTA solution was obtained from Gibco-BRL (Gaithersburg, MD). EHS matrix (commercially available as Matrigel™; Becton Dickinson, Bedford, MA) was prepared in the laboratory according to published protocols [Kleinman et al., 1986] and standardized by determining the protein content with the method of Bradford [1976].

Cell Culture and Drug Treatment

The ECV-304 cell line was obtained from the American Type Culture Collection (ATCC; Reston, VA) and cultured in Medium-199/10% fetal bovine serum (FBS) plus penicillin-streptomycin (Paragon Biotech, Baltimore, MD) for propagation. Cells were rinsed with phosphate-buffered saline (PBS) and passaged with 1× Trypsin-EDTA after 10-min incubation at 37°C. To induce differentiation, a thin layer of EHS matrix (0.8 ml per 10-cm culture dish) was prepared by spreading and gelling at 37°C. Cells were isolated from EHS matrix-coated plates with 2× Trypsin-EDTA treatment for 15–20 min at 37°C. For treatment with chemotherapeutic drugs, 24-well plates were coated with 0.1 ml Matrigel. Cells (5×10^4 per well) were added and cultured in Medium 199/10% FBS or 0.1% FBS with 1–1,000 nM taxol or 0.25–25 μg/ml etoposide.

MTT Assay

After cell incubation, 0.1 ml (96-well plate) or 0.5 ml (24-well plate) of a 2-mg/ml MTT solution in PBS was added to cultured ECV cells in 0.1 ml media (96-well plate) or 0.5 ml media (24-well plate) and incubated for 4 h at 37°C. The medium was discarded and 0.1 ml (96-well plate) or 0.5 ml (24-well plate) of dimethylsulfoxide (DMSO) was added to each well. Absorbance at 595 nm was determined with a BioRad MicroElisa Plate Reader (BioRad, Richmond, CA).

Flow Cytometry

Cells were collected with Trypsin-EDTA and fixed with 70% ethanol. After staining with 50 μg/ml propidium iodide containing 20 μg/ml

RNase A, and 0.1% Triton X-100 in PBS for 20 min, cells were analyzed by fluorescence flow cytometry.

Cytoplasmic and Nuclear Extracts

ECV cells were harvested with Trypsin-EDTA (2× Trypsin-EDTA for Matrigel-cultured cells), rinsed with PBS 3 times, resuspended in hypotonic buffer A (0.2 ml, 10 mM HEPES, pH 7.9, 10 mM KCI, 1.5 mM $MgCI_2$) supplemented with protease inhibitors (leupeptin, aprotinin, each 1 μg/ml; phenylmethylsulfonyl fluoride [PMSF], 0.5 mM), and incubated for 15 min on ice [Johnson et al., 1996]. Cells were lysed by the addition of 25 μl of 2.5% Nonidet P-40 (NP-40)/buffer A and mixed by inversion. Nuclei were pelleted by centrifugation at 500*g* 4°C). The supernatant was recovered (200 μl cytoplasmic extracts), placed into microcentrifuge tubes and stored at −70°C. The nuclear pellet was resuspended in extraction buffer C (20 mM HEPES, pH 7.9, 0.45 M NaCI, 1 mM EDTA) supplemented with proteinase inhibitors and incubated for 15 min on ice on a rocking platform. The tubes were centrifuged (10 min at 16,000*g*). Extracts were diluted 1:1 in buffer D (20 mM HEPES, pH 7.9, 0.1 M KCI, 0.2 mM EDTA, 20% glycerol). The protein concentration was determined before analysis of the samples.

Preparation of Total Protein

Cells were collected with Trypsin-EDTA and lysed with lysing buffer (500 mM Tris, pH 6.8, 10% sodium dodecyl sulfate [SDS], 10% glycerol). Lysed cells were passed through a 25-gauge needle 20 times and centrifuged at 16,000*g* for 30 min to remove debris; the supernatant was stored at −70°C. The protein concentration was determined with the MICRO BCA reagent (Pierce Chemical Co., Rockford, IL).

Electrophoretic Mobility Shift Assay

The consensus κB DNA sequence (5′-GATC-CAGAGGGGACTTTCCGAGAG-3′) was a generous gift from Dr. Chou-Chi H.Li (Frederick Cancer Research Center). The oligo DNA probe was labeled with [γ^{32}P]-ATP and T4 polynucleotide kinase (Pharmacia and UpJohn Diagnostics, Kalamazoo, MI) and separated from free nucleotides over a Sephadex G-25 (STE SELECT-D) spin column (Pharmacia and UpJohn Diagnostics). The reaction solution contained 5 μg nuclear protein, 1 μg poly (dI/dC), 10,000 cpm end-labeled NF-κB oligo probe (5 fmol), 4 μl of 5× reaction buffer (50 mM HEPES, pH 7.9, 5 mM EDTA, 5 mM DTT, 25 μg/ml bovine serum albumin [BSA], 20% glycerol), and distilled H_2O for a final 20-μl volume. The reactants were mixed by gentle inversion and incubated at room temperature for 30 min before loading on a 6% polyacrylamide gel electrophoresis (PAGE)/ 0.5× TBE polyacrylamide gel separated at 150 V/18 cm for 2 h. Gels were dried under vacuum and exposed to X-ray film. Competition experiments were performed as above, except that 10-, 100-, and 1,000-fold excess cold probe was added to the reactions immediately before the addition of [^{32}P]-labeled DNA.

Western Blotting

IκB-α/β. Cytoplasmic extracts that had been frozen in liquid nitrogen were thawed and ultracentrifuged at 100,000*g* for 2 h. Samples (20 μg protein) were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Specific proteins were detected using polyclonal antibodies for IκB-α/β (Biolabs) and horseradish peroxidase (HRP)-coupled secondary antibody (Biolabs) using the ECL method, followed by exposure to X-ray film.

P21/CIP1, Bcl-2, Bax, estrogen receptor. Samples of total protein (20 μg) were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Monoclonal antibodies recognizing P21/CIP1, Bcl-2, or estrogen receptor (Transduction Laboratories, Lexington, KY), polyclonal antibody to Bax (Santa Cruz Biotechnology, Santa Cruz, CA), and (HRP)-coupled secondary antibody (anti-mouse IgG from Transduction Laboratories; anti-rabbit IgG from Biolabs), were used to detect specific proteins on nitrocellulose membranes.

DNA Isolation and Fragmentation

ECV cells were incubated with taxol (50 nM) or etoposide (4 μg/ml) for various lengths of time. Adherent and nonadherent cells were harvested with Trypsin-EDTA and washed with PBS three times. To isolate low-molecular-weight DNA, cell pellets were extracted with 0.6 ml lysis buffer (0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris, pH 7.4) and incubated at 4°C overnight [Caulin et al., 1997]. After centrifugation at 16,000*g* for 30 min to remove nuclei, the supernatant was extracted with phe-

nol-chloroform (3 times), ethanol precipitated, and the low-molecular-weight DNA analyzed on a 1.5% agarose gel in TBE buffer.

Transfection of NF-κB Antisense Oligonucleotides

ECV cells were seeded on 96-well or 6-well plates and incubated overnight (40–50% confluent). Cells were treated with 3 μg/ml antisense NF-κB oligo (5′-GAGGGGGAACAGTTCGTCCATGGCCGGGGT-3′) or sense NF-κB oligo (5′-ACCCCGGCCATGGACGAACTGTTCCCCCTC-3′) prepared by Paragon Biotech. Lipofectin (GIBCO-BRL) at 5 μg/ml in Medium 199 without FBS was used to facilitate oligonucleotide transfer. Cells were incubated for 48 h and transferred to EHS matrix-coated 8-well chamber slides to examine morphological changes indicative of cell differentiation. Incubation with MTT was used to determine viable cells.

RESULTS

Apoptosis of ECV Cells Treated With Taxol and Etoposide: Cell Cycle Analysis and Differentiation

To examine the regulation of EC death, an immortal human EC line, ECV304, isolated from umbilical vein [Takahashi et al., 1990], was treated with the chemotherapeutic drugs taxol and etoposide, to induce cell death. ECV cell survival was reduced by treatment with taxol or etoposide with half-maximal inhibition at 50 nM and 4 μg/ml, respectively (Fig. 1A,B). DNA fragmentation characteristic of apoptosis was detectable on agarose gels in taxol- or etoposide-treated cells (Fig. 1C). Taxol and etoposide treatment produced a pattern of nucleosomal DNA laddering whereas culture of the cells in either 0.1% or 10% FBS did not affect DNA fragmentation or survival. ECV cells were resistant to inhibitors of cell growth such as transforming growth factor-β (TGF-β) or other activators of cell death such as TNF-α (data not shown).

Culture of EC on EHS matrix has been shown to induce growth arrest and differentiation [Kubota et al., 1988; Takahashi et al., 1990; Yang et al., 1996]. ECV cells cultured on EHS matrix migrated to form tube-like structures within 24 h (data not shown). At low cell density ($1.6–3.2 \times 10^5$), essentially all the cells were organized in cellular networks. At higher cell density ($6.4–12.8 \times 10^5$), many of the cells formed monolayers connected by tube-like areas. Fluorescence-activated cell sorting (FACS) was used to determine the distribution of ECV

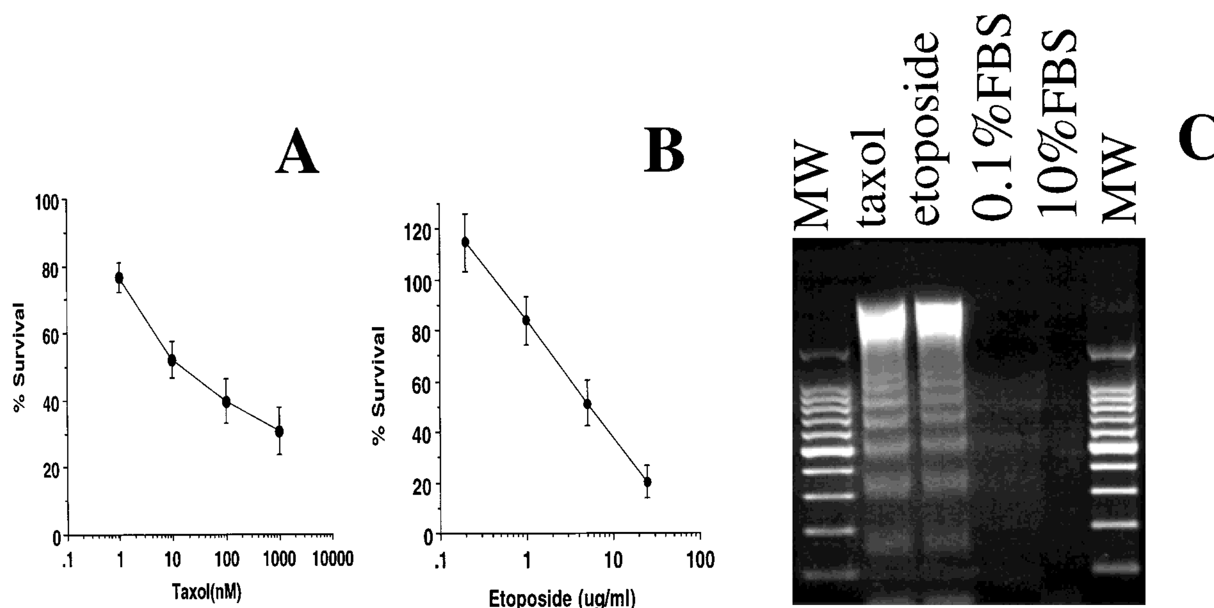


Fig. 1. Taxol and etoposide activate ECV cell apoptosis. Human ECV cells were treated with taxol (**A**) or etoposide (**B**) for 48 h in Medium 199 containing 10% fetal bovine serum (FBS). The MTT substrate was added for 4 h, and the product was quantitated by measuring absorbance at 595 nm. Survival was calculated relative to untreated controls. DNA was isolated from ECV cells treated with taxol (50 nM) or etoposide (4 μg/ml) for 48 h or from untreated control cells in either 0.1% FBS or 10% FBS (**C**). DNA was resolved on an 1.8% agarose gel containing molecular-weight (MW) markers. The nucleosomal-size fragments in taxol or etoposide-treated cells verify the presence of apoptosis.

cells in G1 or S phase of the cell cycle. ECV growth arrest occurred within 48 h on EHS matrix with an increase from 27% to 60% of the cells in G1. The percentage of cells in S phase declined during this period from 72% to 33%. By 72 h, 80% of the cells were growth arrested in G1. By contrast, growth arrest in G1 on tissue culture plastic did not occur until after 72 h with 75–79% of the cells in G1, presumably because of contact inhibition. Therefore, ECV cells are able to sense their extracellular environment and to respond to cell signals from other cells and ECM.

Extracellular Matrix Proteins and Estradiol Inhibit Apoptosis

To determine whether exposure to ECM would affect drug-induced cell death, ECV cells were cultured on EHS matrix or tissue culture plastic for 24, 48, or 72 h in the presence of taxol or etoposide. Cell attachment to ECM enhanced the survival of ECV cells treated with taxol even as early as 24 h (Fig. 2A). Concentrations as high as 1 μM, which inhibited survival up to 70% on plastic, did not reduce ECV survival on EHS matrix. Similarly, culture of ECV on ECM protected the cells against etoposide-induced cell death (Fig. 2B). Significant protection ($P < 0.005$) of ECM was evident at concentrations of etoposide of >1 μg/ml. At 25 μg/ml, about a twofold increase in survival was observed in ECV cells cultured on EHS matrix-coated tissue culture plates, as compared with uncoated plates.

Estrogen is known to have a cardioprotective effect and to inhibit TNF-induced endothelial cell death [Spyridopoulos et al., 1997]. To determine whether ECV cells would respond to estrogen, cells were cultured with 17β-estradiol for 24 h, and cell number was quantitated with the MTT assay. Estrogen had a dose-dependent effect on cell proliferation (Fig. 3A), which was inhibited by the anti-estrogen $ICI_{182,780}$ (data not shown). Consistent with these growth effects, ECV cells grown on plastic or ECM expressed estrogen receptors that were detected by Western blotting, using an estrogen receptor α (ERα)-specific antibody (Fig. 3D). To determine whether estrogen would also protect ECV cells from drug-induced cell death and to compare it with the effects of ECM, ECV cells were pretreated with 17β-estradiol for 24 h before taxol or etoposide treatment for an additional 24 h. Reduction of cell survival by taxol was significantly inhibited by estradiol at all concentrations of taxol (Fig. 3B). This was not due to a

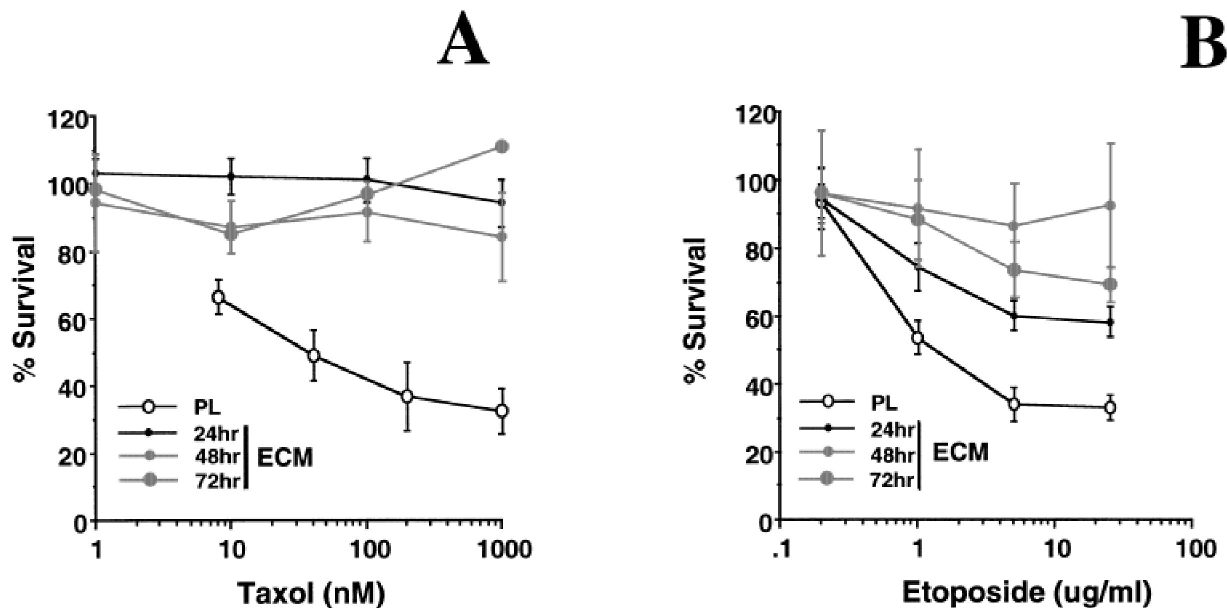


Fig. 2. Culture of ECV cells on the extracellular matrix (ECM) inhibits taxol and etoposide-induced cell death. ECV cells were cultured on tissue culture plates and transferred to either uncoated tissue culture plates (PL) for 24 h or EHS matrix-coated plates (ECM) for 24, 48, or 72 h. Cells were treated with taxol (**A**) or etoposide (**B**) for the last 24 h of the incubation. MTT dye conversion was used to measure percentage survival expressed relative to the absence of drugs. The IC_{50} for taxol or etoposide-treated cells was 40 nM and 1.5 μg/ml, respectively.

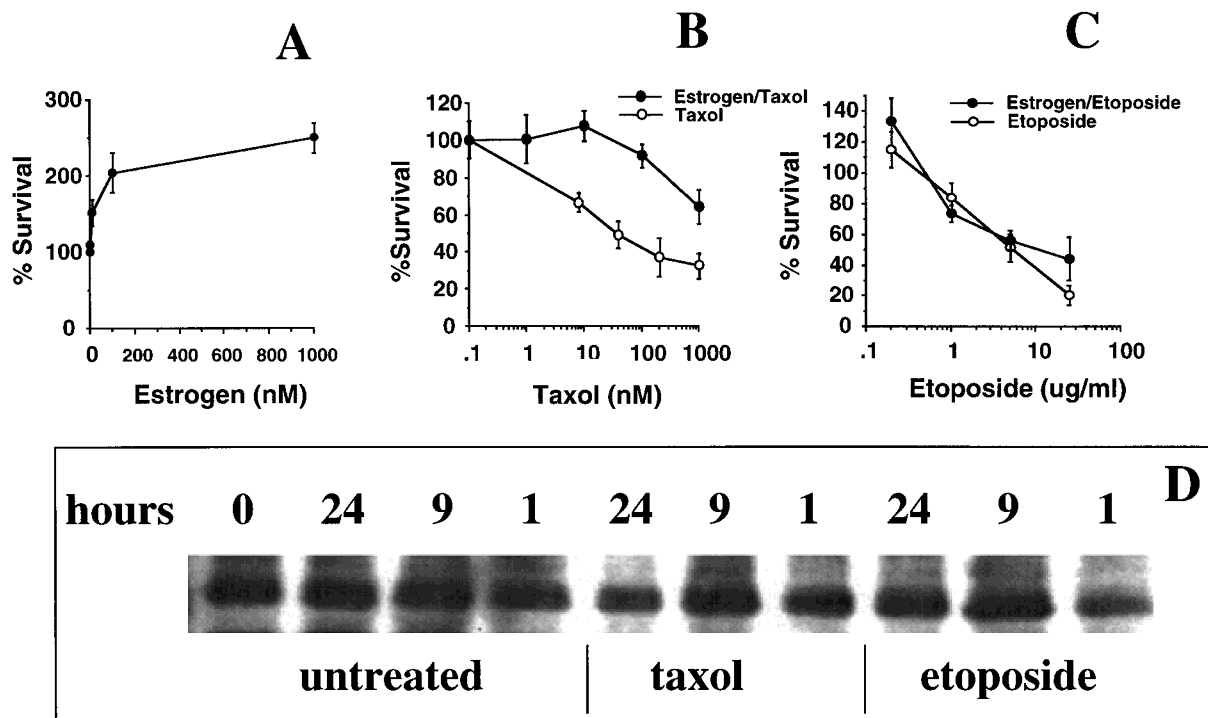


Fig. 3. Estradiol increases ECV cell growth and inhibits taxol-induced apoptosis. ECV cells were cultured on tissue culture plastic in 0.1% fetal bovine serum (FBS) and treated with 17β-estradiol at the indicated concentrations (**A**). The EC_{50} was 45 nM. Cells were pretreated with 17β-estradiol (5 nM) for 24 h before taxol treatment for a subsequent 24 h (**B**). Control cells were treated only with taxol. The IC_{50} for taxol-treated cells was 40 nM. Estradiol treatment 24 h before etoposide treatment (**C**) failed to prevent etoposide-induced cell death. The IC_{50} for etoposide-treated cells was 50 µg/ml. Specific estrogen receptor (ER) antibody was used to determine expression of ER in untreated cells or in cells treated with 50 nM taxol or 4 µg/ml etoposide for 1, 9, or 24 h (**D**). Cells were pretreated for 24 h with 5 nM 17β-estradiol. Drug treatments and controls were in 0.1% fetal bovine serum (FBS).

proliferative effect of estradiol, since at this dose (5 nM) no effect on cell growth was evident (Fig. 3A). By contrast, estrogen failed to protect ECV cells treated with etoposide (Fig. 3C). The failure of estrogen to affect etoposide-mediated apoptosis was not due to loss of estrogen receptors, as similar levels of estrogen receptors were detected in taxol or etoposide treated cells (Fig. 3D).

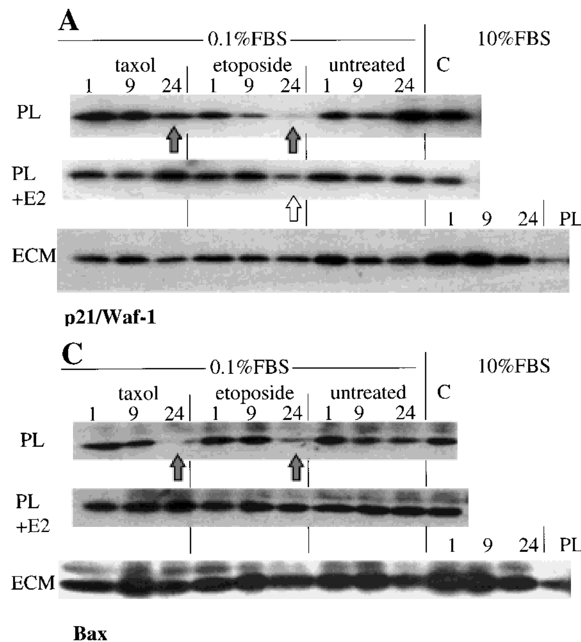
Expression of p21/Waf-1, Bcl-2, and Bax in ECV Cells

Expression of several genes is associated with cellular growth arrest, cell survival, and apoptosis. We examined expression of the p53-inducible p21/Waf-1 protein, the cell survival-enhancing protein Bcl-2, and the cell death-associated protein, Bax, in ECV cells treated with taxol or etoposide. Cells were cultured on plastic or ECM (EHS matrix) with or without prior estradiol treatment. p21 protein levels declined by 24 h in cells on plastic treated with taxol or etoposide (Fig. 4A, solid arrows). The reduction in p21 was prevented by estradiol treatment in taxol-treated cells, but not in etoposide-treated cells (Fig. 4A, open arrow). Culture on ECM prevented down-regulation of p21 in both taxol- or etoposide-treated cells. Similarly, Bcl-2 levels declined most significantly in etoposide treated cells (Fig. 4B, arrows) and estradiol prevented this decline. Bax levels were reduced in both taxol- and etoposide-treated cells (Fig. 4C, arrows), an effect that was prevented by estradiol treatment.

Therefore, changes in p21 are most consistent with the ability of estrogen to prevent taxol-mediated, but not etoposide-mediated, cell death, and the ability of ECM to prevent reduction of p21 is consistent with the protective effects of ECM on cell survival.

NFκB in Differentiation and Survival

Activation of NFκB occurs in inflammation [Lenardo and Baltimore, 1989], in the differen-



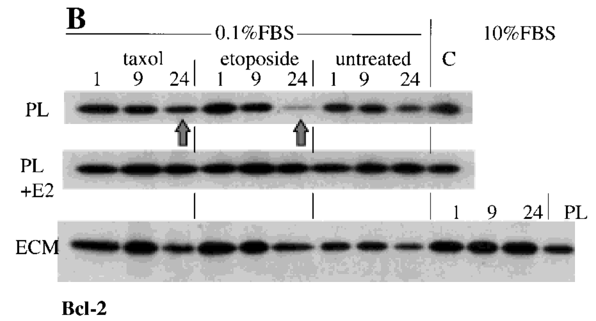


Fig. 4. Taxol and etoposide-induced cell death results in decreased levels of p21, Bcl-2, and Bax. ECV cells were prepared as in Figure 5. p21, Bcl-2, and Bax were detected by Western blot analysis, using specific antibodies. Estradiol prevents reduction of p21 (**A**), Bcl-2 (**B**), and Bax (**C**) after taxol and etoposide treatment (solid arrows), but not the reduction of p21 after etoposide treatment (open arrow) consistent with survival data in B. Culture of ECV cells on EHS matrix (ECM) prevented the p21, Bcl-2, and Bax down-regulation induced by taxol or etoposide, consistent with increased survival.

tiation of endothelial cells [Johnson et al., 1996], and in cell survival [Van Antwerp et al., 1998; Beg and Baltimore, 1996]. We therefore examined the ability of ECV nuclear extracts to bind specific NFκB oligonucleotides in an EMSA to assess the role of NFκB in ECV survival and differentiation. ECV cells grown on plastic exhibited fairly low levels of NFκB activity in either 0.5% FBS (Fig. 5A) or in 10% FBS (not shown). TNF-α treatment in 0.5% FBS resulted in persistent NFκB activation. Similarly, transfer of cells to ECM (Matrigel) resulted in a rapid (1-h) elevation of NFκB activity that persisted for 24 h in cells maintained in 0.5% FBS or 10% FBS (Fig. 5A). Control experiments with excess unlabeled oligonucleotides prevented nuclear factor binding (Fig. 5B). In other experiments with cultures of primary human umbilical vein endothelial cells that respond to TNF-α, culture on Matrigel also resulted in persistent activation of NFκB, whereas TNF-α treatment transiently activated NFκB (data not shown). Therefore, persistent activation of NFκB by ECM is a feature of both primary and immortalized endothelial cells. However, examination of EMSA in cells treated with taxol, etoposide, or estradiol exhibited no changes in NFκB activity (data not shown).

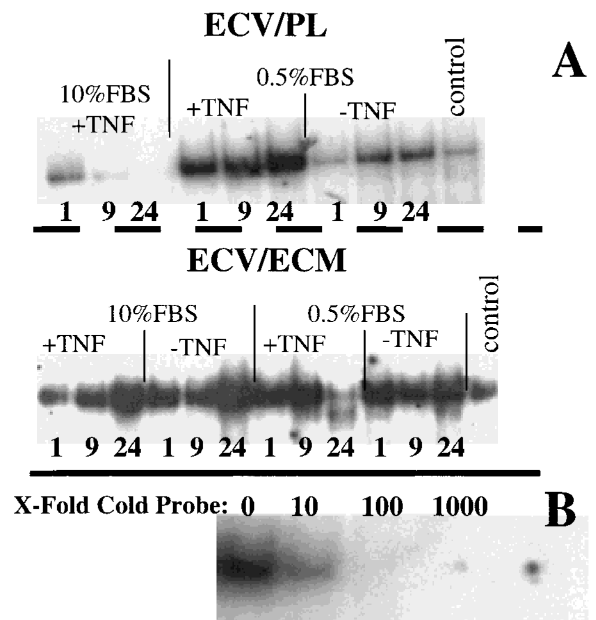


Fig. 5. Persistent activation of NFκB is coincident with attachment of ECV cell to EHS matrix (ECM) and differentiation. **A:** ECV nuclear extracts were prepared at different times, reacted with radiolabeled NFκB oligonucleotides and resolved on 6% TBE polyacrylamide gels. Specific radiolabeled NFκB oligonucleotide probe was used to detect nuclear binding proteins. **B:** Addition of 10- to 1,000-fold excess unlabeled κB oligonucleotides inhibited bandshifting. ECM, EHS matrix; PL, plastic.

IκB Regulation in ECV Treated With Taxol and Etoposide

Activation of the nuclear factor-κB is regulated by the expression of two inhibitors, IκBα and IκBβ. Normally, proteolytic degradation of IκB leads to high levels of NFκB proteins and to increased NFκB DNA binding activity [Siebenlist et al., 1994]. To determine whether the persistent activation of NFκB observed in ECV

cells cultured on ECM was regulated by IκBα or IκBβ, and whether the levels of these proteins were altered in cells undergoing apoptosis, ECV cells treated with taxol or etoposide were analyzed after culture on ECM. IκBα expression was unchanged by taxol treatment but declined in etoposide-treated cells, coincident with cell death (Fig. 6A, solid arrows). Estradiol treatment did not prevent the reduction of IκBα (Fig. 6A, PL + E2) and failed to protect cells against etoposide-mediated apoptosis. Consistent with the prevention of ECV cell death on ECM and with elevated levels of NFκB, IκBα levels in etoposide-treated cells on Matrigel were lower than levels in untreated cells after 24 h (Fig. 6A, open arrows).

Interestingly, IκBβ levels (Fig. 6B, arrows) were lower than control levels only in etoposide-treated cells on ECM. No changes in IκBβ were evident in cells treated with drugs or estradiol on tissue culture plastic. This finding suggests that normal regulation of IκBα and IκBβ occurs in etoposide-treated cells. NFκB levels do not correlate with survival on plastic, but persistent activation of NFκB on ECM may be regulated by expression of IκBα and IκBβ.

Differentiation and Antisense NFκB Oligonucleotides

Because culture of cells on ECM may activate a variety of signaling pathways affecting both cell survival and differentiation, ECV cells were transfected with specific NFκB antisense oligonucleotides to determine whether the NFκB pathway was involved in differentiation. Transfected cells were incubated for 48 h before transfer to ECM-coated plates. ECV cell attachment and migration were not affected by any of the treatments during the first 24 h (Fig. 7A). By 36 h, however, antisense transfected cells exhibited retraction of tube-like structures and increased cell aggregation (Fig. 7B). This inhibition of differentiation was quantitated by measuring the percentage cell area in each of the treatment groups. The percentage cell area relative to total area of the field in untransfected (control), lipofectin-treated, and sense oligonucleotide-treated cells remained at 14.2 ± 0.7, 13.5 ± 1.9, and 15.2 ± 2.2, respectively, while antisense-treated percentage cell area decreased by 66% to 4.2 ± 0.9 (Fig. 7C). After incubation on ECM, cells were treated with MTT to measure cell viability. No evidence of cell death was detected, and equal numbers of cells were recovered from all treatment groups even 96 h after incubation on ECM (data not shown). Therefore, NFκB antisense oligonucleotides inhibited ECV cell differentiation without affecting cell growth or survival.

DISCUSSION

We have found that EC apoptosis occurs after treatment of ECV cells with the chemotherapeutic drugs taxol or etoposide. Culture of the cells on ECM inhibited apoptosis induced by taxol or etoposide, whereas treatment with estradiol inhibited apoptosis induced by taxol only. Apoptosis was most closely associated with loss of p21 expression after taxol or etoposide treatment. Persistent activation of NFκB was protective against apoptosis but did not consistently correlate with reduced levels of IκB, except in etoposide-treated cells cultured on ECM. Under these

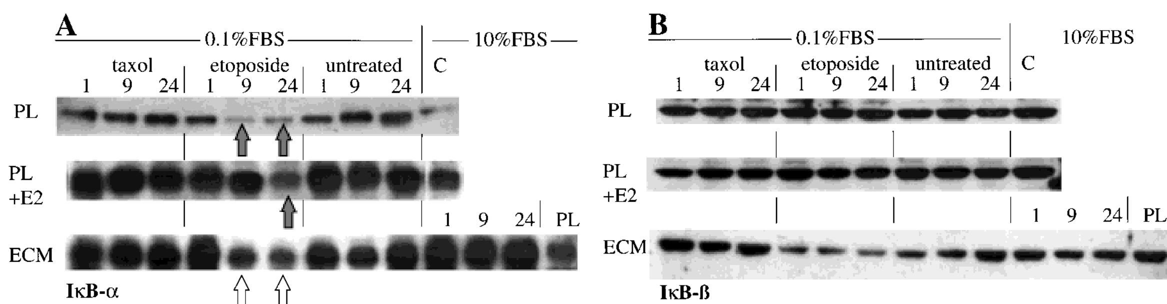


Fig. 6. IκB expression in taxol- or etoposide-treated ECV cells. Western blot analysis was used to determine cytoplasmic protein levels of IκBα (**A**) or IκBβ (**B**) in ECV cells treated with taxol or etoposide. IκBα levels are persistently elevated in taxol-treated cells but are transiently elevated in etoposide-treated cells (solid arrows). Estrogen did not prevent reduction of IκBα in etoposide-treated cells (arrow). Culture on extracellular matrix (ECM) increased IκBα transiently in etoposide-treated cells (open arrows). IκBβ levels did not change, except to decline in etoposide-treated cells on ECM consistent with elevated NFκB and survival.

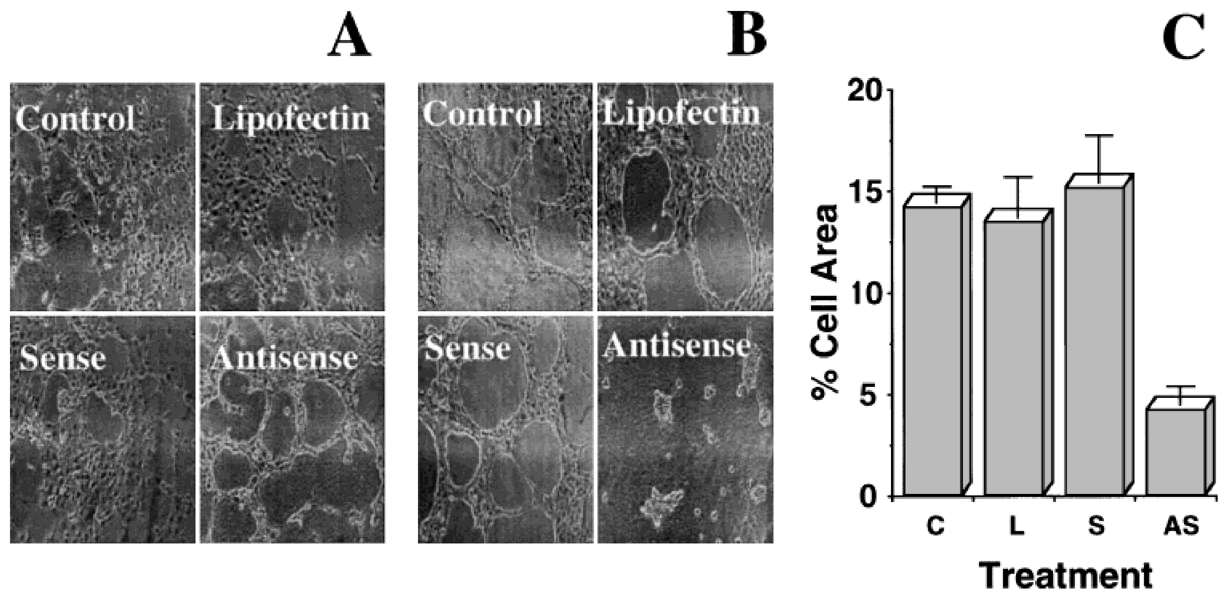


Fig. 7. Transfection of ECV cells with anti-sense NFκB oligonucleotides inhibits ECV differentiation on extracellular matrix (ECM). ECV cells were transfected with lipofectin (L), lipofectin + anti-sense (AS), or lipofectin + sense (S) or were not transfected (control, C). After 48 h, cells were transfered to EHS matrix-coated plates and observed for morphological changes. In vitro differentiation was observed for all treatments after 6–24 h on EHS matrix (**A**). However, by 36 h, networks of ECV transfected with antisense NFκB oligos began to retract, and differentiation was inhibited (**B**). Network area was quantitated at 36 h, using a video camera and Image-1 analysis software (**C**).

conditions culture on ECM resulted in decreased levels of IκBα and IκBβ with persistent activation of NFκB and increased cell survival. Therefore, normal regulation of IκBα,β/NFκB occurred in etoposide-treated cells on ECM. Elevated NFκB did not correlate with survival on plastic but did correlate with survival on ECM. Lastly, NFκB activation in ECV cells cultured on ECM was found to be more important in cell differentiation than in cell survival, since antisense NFκB oligonucleotides inhibited cellular differentiation without affecting cell survival.

Huvec have been used for many studies of EC function [Kubota et al., 1988; Morales et al., 1995; Yang et al., 1996]. However, as primary isolates, these cells exhibit a limited life span in culture and do not survive most transfection protocols. ECV304 cells were isolated from a culture of primary Huvec that had undergone spontaneous immortalization [Takahashi et al., 1990]. Our studies confirm that ECV cells exhibit growth arrest on Matrigel with associated morphological differentiation. Other EC cells have been shown to respond to estradiol and express estrogen receptors [Venkov et al., 1996; Spyridopoulos et al., 1997; Morales et al., 1995]. We report that ECV also express ERα and that estradiol has a growth-promoting effect that is inhibited by antiestrogen. These results are important because they indicate that these immortalized cells still retain hormonal responses and may be useful targets to study the effects of estrogen antagonists on endothelial cell function. In our study, we also found that ECV will undergo apoptosis in response to the chemotherapeutic drugs taxol or etoposide. Interestingly, ECM could prevent drug-induced apoptosis. This could be attributable to survival signals mediated through ECM/integrin binding [Meredith et al., 1996] or to the nonresponsive state of the cells in G1 growth arrest [Bhalla and Harris, 1998]. In addition, the presence of growth factors in EHS matrix may contribute to the observed protective effects on cell death. Estradiol was able to inhibit the effects of taxol on ECV cells but was not effective in preventing etoposide-mediated apoptosis. Taxol acts to stabilize microtubules and to prevent cell division [Jordan and Wilson, 1998], while etoposide is a topoisomerase inhibitor acting on enzymes involved in DNA replication [Austin and Marsh, 1998]. It is possible that the protective effects of estradiol are most evident in cells in growth arrest (taxol, ECM treatment) and that normally cycling cells (etoposide-treated) are resis-

tant to its effects. As with most hormone receptors, transcriptional activation of other genes involved in cell survival may mediate the protective effects of estradiol.

The apoptosis regulating proteins, Bcl-2 and Bax, have been shown to function as heterodimers with relatively high expression of Bcl-2 supporting cell survival and high expression of Bax inducing apoptosis [Granville et al., 1998]. In ECV cells, levels of Bcl-2 and Bax declined in cells undergoing apoptosis in the presence of taxol or etoposide. Either ECV or estradiol could prevent this down-regulation. However, estradiol could not protect ECV cells against etoposide-mediated cell death, even though Bcl-2 and Bax levels remained high. It is possible that other bcl-2 or Bax family members may be involved in apoptosis in these cells. On the other hand, p21 expression declined in ECV cells treated with etoposide, even in the presence of estradiol. This finding suggests that failure to maintain adequate levels of p21 protein is incompatible with survival. By contrast, cells cultured on ECM in the presence of etoposide did not undergo apoptosis while maintaining high levels of p21 expression. These results are consistent with our previous observations using growth factor-deprived Huvec [Yang et al., 1995]. Therefore, it appears that in these ECV cells, regulation of apoptosis may depend more on the cell cycle inhibitor p21 than on Bcl-2/Bax ratios. In agreement with these findings, it has been reported that expression of p21 is important in differentiation and survival [Halevy et al., 1995; Parker et al., 1995]. In future studies, overexpression of p21 in ECV cells may be one way to address this issue.

NFκB activity was persistently elevated in ECV cells cultured on ECM. These results suggested that NFκB activity was associated with either cell differentiation or cell survival. To show that NFκB activation was mediating these effects directly, specific NFκB antisense oligonucleotides were used to transfect ECV. Although cell differentiation was completely inhibited by this treatment, cell viability was unaffected. These results are consistent with reports that NFκB is activated in fibroblasts cultured on collagen matrix [Xu et al., 1998] and that NFκB enhances differentiation of microvascular EC in response to hydrogen peroxide and TNF-α [Shono et al., 1996]. Normally, IκB levels regulate NFκB activity with phosphorylation of IκB protein and subsequent degradation by proteasomes leading to enhanced NFκB activity [Stancovski and Baltimore, 1997]. However, ECV cells cultured on ECM exhibited high NFκB activity without loss of IκBα or -β. Similar studies have been reported using B cells [Phillips and Ghosh, 1997] in which NFκB DNA binding complexes were found in the nucleus, even in the presence of high levels of IκBβ in the cytosol. However, other studies using Huvec have reported that persistent NFκB activation by TNF-α results from a sustained reduction in IκBβ levels [Johnson et al., 1996]. The only conditions in which we observed reduction of IκB levels with subsequent NFκB activation were in etoposide-treated cells on ECM. Under these conditions, growth arrest and possible survival signals may lead to degradation of IκB while preventing apoptosis.

In conclusion, we have shown that in immortalized ECV cells, apoptosis signal transduction is regulated by p21 expression, and to some extent by estradiol and NFκB activation, in etoposide-treated cells. NFκB activity was particularly important in regulating ECV differentiation. The results suggest that these cells may be useful to study the role of chemotherapeutic agents and hormones in EC apoptosis and differentiation.

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