Journal of Cellular Biochemistry 85:728–736 (2002)

# Soluble Osteopontin Inhibits Apoptosis of Adherent Endothelial Cells Deprived of Growth Factors

S.A. Khan,[1] C.A. Lopez-Chua,[1] J. Zhang,[1] L.W. Fisher,[2] E.S. Sørensen,[3] and D.T. Denhardt[1]*

[1]Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, New Jersey 08854
[2]Craniofacial and Skeletal Diseases Branch, NIDCR, National Institutes of Health, Bethesda, Maryland
[3]Protein Chemistry Laboratory, University of Aarhus, Science Park, DK-8000 Aarhus C, Denmark

**Abstract** Osteopontin (OPN) is primarily an extracellular glycosylated phosphoprotein capable of stimulating cell migration and cell attachment, predominantly to mineralized surfaces. Found in moderate levels in plasma, it acts as a cytokine able to modify gene expression via integrins and certain CD44 isoforms. In this work we show that soluble OPN inhibits apoptosis of adherent human umbilical vein endothelial cells incubated in medium lacking critical growth factors and cytokines. In a dose-dependent manner OPN reduced the formation of apoptotic bodies and suppressed DNA fragmentation. OPN also caused an increase in Bcl-$X_L$ mRNA levels, suppressed the apparent dispersion of Bcl-$X_L$ throughout the cytoplasm, and slightly enhanced IκB-α protein degradation. These data suggest that a function of OPN in homeostatic processes is to facilitate the survival of stressed endothelial cells, possibly by occupying unligated integrins and suppressing integrin-mediated death. J. Cell. Biochem. 85: 728–736, 2002. 

**Key words:** Bcl-$X_L$; programmed cell death; DNA fragmentation; integrin-mediated cell death

Osteopontin (OPN), one of the major non-collagenous proteins in bone, is also found in body fluids, where it appears to have properties of a cytokine in that it can modify cellular activities by interacting with certain integrins and the hyaluronic acid receptor CD44. Although primarily a secreted, phosphorylated glycoprotein, there is evidence also for an intracellular form associated with CD44 and ezrin-radixin-moesin proteins [see review Sodek et al., 2000]. Several observations suggest that OPN signaling may regulate or moderate the inflammatory response incurred during infection or injury [see review Denhardt et al., 2001]. Well-established functions include the ability to inhibit the induction of inducible nitric oxide synthase, to attract macrophages, and to enhance the metastatic properties of transformed cells [Hwang et al., 1994; see reviews in Giachelli et al., 1995; Oates et al., 1997]. As an adhesion protein, OPN can increase the resistance of osteoligament and aortic endothelial cells to heat shock [Sauk et al., 1990] and serum deprivation [Scatena et al., 1998], respectively, promoting the survival of these cells under the given stress. As a cytokine, OPN also promotes the survival of proximal tubule epithelial cells exposed to hypoxia-reperfusion injury [Denhardt et al., 1995; Noiri et al., 1999] and stimulates cell-mediated auto-immune processes [Ashkar et al., 2000; Chabas et al., 2001].

Evidence that OPN is not essential for normal developmental or reproductive processes comes from the observation that mice unable to make OPN develop normally, have apparently normal bones and teeth, and are fertile [Rittling et al., 1998; Liaw et al., 1998; Rittling and Denhardt, 1999]. Interestingly, and in contrast to normal mice, the bones of mice unable to make OPN do not lose calcium upon ovariectomy-induced estrogen deprivation [Yoshitake et al., 1999].

Abbreviations used: BSA, bovine serum albumin; ECGS, endothelial cell growth supplement; ECM, extracellular matrix; FGF, fibroblast growth factor; HUVECs, human umbilical vein endothelial cells; b, h, or mOPN, bovine, human, or mouse osteopontin; PBS, phosphate-buffered saline, calcium-free.

Grant sponsor: NIH; Grant numbers: AR44434, ES06897.

S.A. Khan and C.A. Lopez-Chua contributed equally to the research presented here.

*Correspondence to: D.T. Denhardt, Nelson Laboratories, 604 Allison Rd., Piscataway, NJ 08854.
E-mail: denhardt@biology.rutgers.edu

Received 8 February 2002; Accepted 11 February 2002
DOI 10.1002/jcb.10170

Resorption of ectopic bone [Asou et al., 2001] and disuse osteoporosis [Ishijima et al., 2001] are also deficient in the OPN-null mice. The research reported herein had its origin in the observation by Lopez et al. [1996] that OPN could inhibit heregulin-induced apoptosis of the breast cancer cell line SKBr3. In this study we show that soluble OPN can suppress apoptosis of human umblical vein endothelial cells (HUVECs) deprived of growth factors. This is consistent with the emerging appreciation that certain receptors (e.g., $p75^{NTR}$, the androgen receptor, and DCC) will induce apoptosis if they are not engaged by their ligands [Bredesen et al., 1998; Mehlen et al., 1998]. Results described below suggest that OPN acts as a cytokine to inhibit the induction of apoptosis in the factor-deprived HUVECs by engaging unoccupied "dependence receptors," possibly an integrin or CD44.

## MATERIALS AND METHODS

### Cell Culture

Primary human umbilical vein endothelial cells (HUVECs) (Clonetics, San Diego, CA) were routinely cultured on gelatin (porcine Type A, Sigma, St. Louis, MO)-coated plates in Medium 199 (Life Technologies, Rockville, MD), supplemented with 10 mM L-glutamine, 10% fetal bovine serum (Hyclone, Logan, UT), heparin (5 U/ml; Sigma), endothelial cell growth supplement (ECGS, 7.5 μg/ml; Sigma), and αFGF (4 ng/ml; Sigma) [Re et al., 1994]. Cells were maintained at 37°C in 5% $CO_2$ and passaged by detaching with 0.05% trypsin (Gibco-BRL, Life Technologies). Viability was measured by Trypan Blue exclusion. In all cases, when cells were cultured in the absence of added factors (serum, heparin, glutamine, ECGS, and αFGF), 1% bovine serum albumin (BSA, Sigma) was added, regardless of the presence or absence of OPN. Bovine and human OPN was purified from milk or from cell culture medium as described by Sørensen and Petersen [1993] and Hwang et al. [1994], respectively. Recombinant human osteopontin was also made by amplifying a full-length human OPN cDNA with oligonucleotides containing *Asp*718 (5′) or *Bam*HI (3′) restriction sites immediately outside the coding domain. The PCR product was gel purified, digested with *Asp*718 and *Bam*HI and ligated into a pACCMV.pLpA plasmid prepared with the same enzymes. A single clone (OF) was isolated and sequenced. The plasmid was used in a double recombination event to construct a replication-deficient adenovirus in a strain of replication-supporting 293 cells. The virus was plaque purified and amplified to high titer in the same 293 cells. Recombinant protein was made by infecting human marrow fibroblasts and purifying the protein to greater than 95% pure as previously described [Fedarko et al., 2000]. On those occasions when the different preparations of OPN were tested, comparable results were obtained.

### Acridine Orange Staining

Subconfluent HUVECs (passage 13, $5 \times 10^5$ cells/10-cm dish) were washed three times with phosphate-buffered saline (PBS) and incubated for 24 h in M199 medium supplemented with factors as above or with 1% BSA supplemented or not with the indicated concentrations of hOPN. The cells were trypsinized and collected by centrifugation (3,000*g*, 10 min); floating cells and debris were similarly pelleted from the conditioned medium. The pellets were combined, washed twice with cold PBS, and fixed in 70% ethanol for 10 min at 4°C. The fixed cells were washed in PBS and suspended in 50 μl of PBS and spread on poly-L-lysine-coated slides, dried, and stained for 3 h with acridine orange (3 μg/ml in PBS). The slides were washed, mounted with DABCO, and photographed with an FITC filter [Polunovsky et al., 1994].

### DNA Fragmentation Assessment

HUVECs were cultured as described above in the presence or absence of factors. After the indicated times of factor deprivation (+ or − OPN) both the adherent and non-adherent cells were harvested by trypsinization and centrifugation. The cells were washed with cold PBS and then incubated in Nicoletti lysis buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS, and 50 μg/ml proteinase K) at 43°C overnight. The lysate was extracted with buffered phenol followed by chloroform/isoamyl alcohol. The aqueous phase was incubated for 1 h at 37°C with 50 μg/ml RNase and then extracted with chloroform/isoamyl alcohol. DNA was precipitated with 0.5 M NaCl and 66% ethanol. The DNA was redissolved in 50 μl of water and its concentration determined by spectrophotometry. Each DNA sample (10 μg/lane) was electrophoresed in a 2% agarose gel in

a tris-acetate-borate buffer. The DNA in the gel was stained with ethidium bromide and photographed under ultraviolet radiation.

### Staining for Bcl-$X_L$ and Mitochondria

Acid-washed 12 mm-coverslips were coated 1% gelatin, which was then crosslinked with 1% glutaraldehyde in PBS. HUVECs were plated ($4 \times 10^4$/well) on these coverslips in a 24-well tissue culture dish. Two days later, cells were deprived of factors and supplemented with 1% BSA, plus or minus bOPN (5 or 500 pM). MitoTracker dye (MitoTracker Red CMSRos; Molecular Probes, Eugene, OR) was diluted to a final concentration of 100 nM in M199 medium (supplemented with factors or with only 1% BSA). After 12 h of factor deprivation, the medium was replaced with M199 medium containing MitoTracker dye and cells were incubated at 37°C for 20 min. At the end of incubation, the dye-containing medium was aspirated and cells were washed three times with PBS followed by fixing with 3.7% paraformaldehyde for 10 min at 37°C. Fixed cells were again washed three times with PBS and then permeabilized with 0.3% Triton X-100 + 5% BSA for 1 h at room temperature. Cells were incubated for 1 h at room temperature with anti-Bcl-$X_L$ antibody (1 μg/ml; Santa Cruz Biotech, Santa Cruz, CA), washed three times in PBS, and incubated with FITC-conjugated Affinipure goat anti-rabbit IgG (H + L) (1:100 dilution; Jackson Immuno Research Laboratories, West Grove, PA) for 1 h at room temperature. The coverslips were then washed three times with PBS and mounted in Prolong Anti Fade mounting medium (Molecular Probes). The cellular localizations of Bcl-$X_L$ and mitochondria were visualized using a Zeiss Laser Scanning LSM 510 microscope at 40 × magnification.

### Analysis of Bcl-$X_L$ Expression by Reverse Transcription Polymerase Chain Reaction (RT-PCR)

HUVECs (passage 7) in M199 medium were deprived of factors as described above, with or without hOPN (50 nM) for 1, 3, and 12 h. Cellular RNA was isolated by the TRIzol RNA isolation procedure (Life Technologies). A quantitative RT-PCR was carried out to quantify the abundance of Bcl-$X_L$ mRNA relative to 18s rRNA (Ambion, Austin, TX). Bcl-$X_L$ primers (5′-TTGGACAATGGACTGGTTGA-3′ and 5′-CATCTCCTTGTCTACGCTTTC-3′) were designed according to human Bcl-$X_L$ c-DNA sequence. Primers for 18S rRNA were from Ambion. Amplification was performed at 94°C for 1 min, 51°C for 1 min, and 72°C for 1 min. Bcl-$X_L$ was amplified for 23 cycles and 18S rRNA for 15 cycles, based on preliminary experiments to determine the linear range.

### IκB-α Analysis

Immunoblot analysis was performed following the protocol (with some modifications) of PhosphoPlus IκB-α (Ser 32) antibody kit (New England BioLabs, Beverly, MA) to detect expression of IκB-α protein in OPN-treated and control HUVECs. Briefly, HUVECs were cultured as described and treated with OPN (50 nM) for 1, 2, 4, or 6 h. After each time point, medium was aspirated from culture and the cells were washed with ice-cold 1% PBS. Cells were lysed by adding 100 μl of SDS sample buffer and immediately scraped off the plate. The cell extracts were transferred to a microcentrifuge tube and sonicated for 10–15 min on ice. Samples were centrifuged and the resultant supernatants analyzed for protein using the Bradford reagent. Twenty micrograms of protein were boiled and electrophoresed on 15% SDS–polyacrylamide gels. Proteins were transferred onto a polyvinylidene fluoride membrane and blocked overnight with 5% dry milk in 1 × Tween-20-Tris-buffered saline. The membrane was incubated with polyclonal rabbit anti-IκB-α antibody for (1:1,000) 1 h at 4°C. After washing, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:3,000) for 1 h at 4°C. Proteins were detected using the LumiGLO chemiluminescent reagent provided with the kit.

## RESULTS

The removal of growth factors and cytokines from adherent human umbilical vein endothelial cells induces a form of apoptotic cell death. The addition of soluble OPN to the factor-deprived cells reduces the amount of cell death in a dose-dependent manner. Figure 1 shows by Trypan Blue staining that there was a fourfold increase in the number of dead cells by 24 h, and that OPN at concentrations of 0.1 nM or more suppressed most of this cell death. (Normal human plasma levels of OPN are about 2 nM, however the OPN is mostly complexed with Factor H and consequently rendered inaccessible

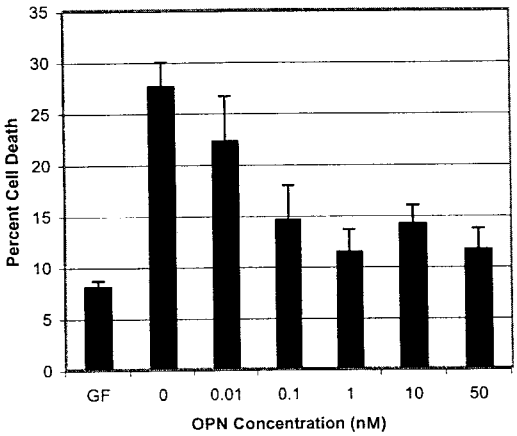


**Fig. 1.** Osteopontin (OPN) promotes the survival of growth factor/cytokine-deprived HUVECs. Cells incubated for 24 h in complete medium (GF, leftmost bar) or in medium lacking factors but with 1% BSA and increasing concentrations of hOPN (nM). The percent surviving cells was determined by Trypan Blue exclusion.

to cell surface receptors [Fedarko et al., 2000]). Cells stained with acridine orange are illustrated in Figure 2. A substantial increase in ghost-like apoptotic vesicles is apparent in the absence of factors (compare 2A and 2B); 100 nM hOPN (2D), but not 1 pM hOPN (2C), suppressed most of this apoptosis. The ability of OPN to inhibit the DNA breakdown characteristic of apoptotic cells is evident in Figure 3. In a concentration-dependent manner, OPN suppressed the fragmentation of the cellular DNA characteristic of the apoptotic process.

As a first step to discover mechanisms underlying how OPN might inhibit apoptosis induced by deprivation of factors, we investigated the impact of OPN on the abundance and distribution of members of the Bcl-2 family of pro- and anti-apoptotic proteins. Although no change in Bcl-2 itself was detected (data not shown), we

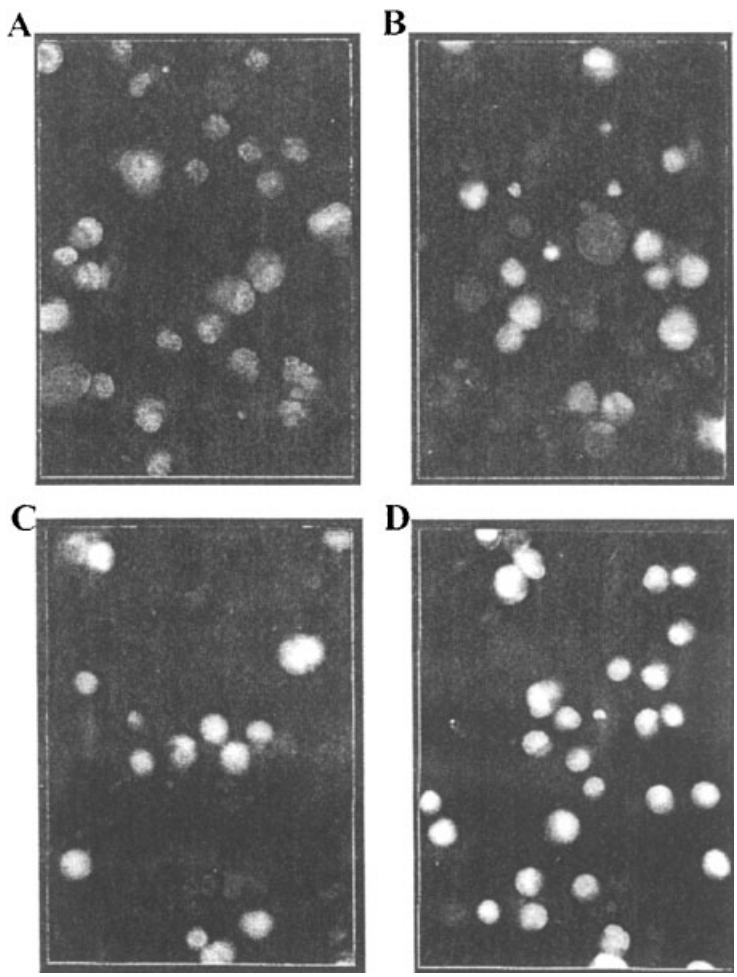


**Fig. 2.** OPN suppresses apoptosis of factor-deprived HUVECs. Cells were stained with acridine orange as described in Materials and Methods. **A**: Control cells; (**B**) growth factor/cytokine-deprived cells; (**C** and **D**) are cells in the absence of growth factors and cytokines but with 1 pM and 100 nM hOPN, respectively.

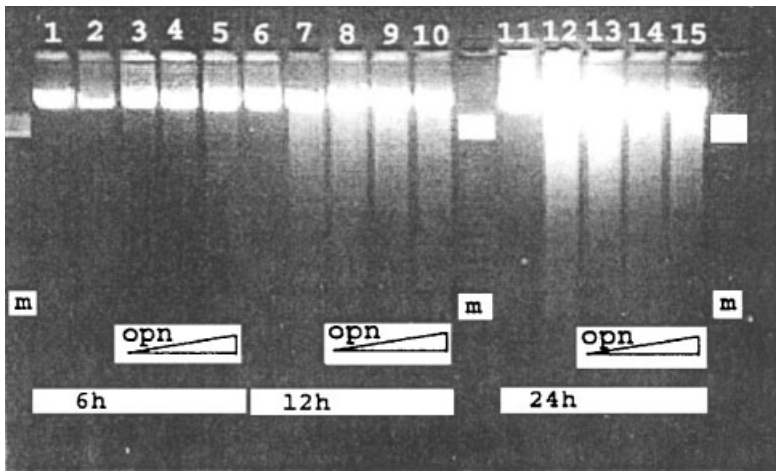


**Fig. 3.** OPN inhibits DNA breakdown in factor-deprived HUVECs. DNA was prepared from cells and analyzed in agarose gels as described in Materials and Methods. Cells were incubated for 6 h (**lanes 1–5**), 12 h (**lanes 6–10**) or 24 h (**lanes 11–15**) in the presence (1, 6, 11) or absence (remaining lanes) of factors. Lanes 3, 8, and 13 contained 0.5 nM hOPN; lanes 4, 9, and 14 contained 5 nM hOPN; and lanes 5, 10, and 15 contained 50 nM hOPN. M, markers. The marker bands and the ladder structure of the apoptotic DNA in lanes 7–9 and 12–14 are visible on the original photograph.

did observe both a change in the abundance and apparent distribution of the Bcl-2 family protein Bcl-$X_L$ in the cell. Bcl-$X_L$, the "anti-apoptotic" isoform of the Bcl-$X_{s/l}$ protein, appears to function by inhibiting activation of procaspases and maintaining the integrity of organelle membranes [Adams and Cory, 1998]. Results presented in Figure 4 show that OPN affects the distribution of Bcl-$X_L$ in the cell. In contrast to the control cells in Figure 4A, the factor-deprived cells in Figure 4B are undergoing apoptosis and exhibit a change in the distribution of Bcl-$X_L$ from a predominantly perinuclear location to a more diffuse cytoplasmic distribution. (Whether there is an actual change in the distribution, or a change in the staining efficiency of the Bcl-$X_L$ protein in the different locations cannot be distinguished by these data.) Two different concentrations, 5 and 500 pM (Figures 4C,D), of bOPN inhibited apoptosis and the apparent redistribution of Bcl-$X_L$. Similar results were obtained with hOPN [Denhardt and Noda, 1998]. In cells undergoing apoptosis (Fig. 4B,C) Bcl-$X_L$ staining become much more prominent, especially over the nuclei, reflecting the tendency of the cells to round up. The mitochondria, which stain red in the absence of Bcl$X_L$, appeared to retain the perinuclear localization both in the control cells and in the apopotic cells regardless of the presence of OPN. The orange stain indicates that Bcl$X_L$ colocalizes with the mitochondria almost entirely in panels A and D, whereas in the apoptotic cells, panels B and C, there is significant Bcl$X_L$ not colocalized with the mitochondria.

To evaluate the Bcl-$X_L$ expression level in OPN-treated, factor-deprived HUVECs a quantitative RT-PCR analysis was carried out. After exposure to 50 nM hOPN for 12 h, Bcl-$X_L$ mRNA levels increased by 50% compared to the control cells (Fig. 5). Despite the increased mRNA levels, the abundance of Bcl-$X_L$ protein appeared unchanged (data not shown).

To obtain some insight into the nature of the signaling pathway that OPN stimulates, we investigated the phosphorylation of selected proteins. There was either no detectable change (Akt) or small and transient changes (cdc2, cdk2, fos) of uncertain significance (data not shown). There was, however, a noticeable and reproducible degradation of of IκB-α upon OPN treatment, as shown in Figure 6. A decreased level of IκB-α level would increase NF-κB translocation to the nucleus and hence upregulate of transcription of NF-κB-controlled genes, for example Bcl-$X_L$.

## DISCUSSION

Cells may die for various reasons. Signals provided to adherent cells by the extracellular matrix (ECM) are required for cell survival. Components of the ECM (e.g., fibronectin, collagen) interact with cell surface receptors (notably various integrins) to activate requisite anti-apoptotic signal transduction pathways [Schwartz, 2001]. Vitronectin, but not fibronectin, can protect adherent glioma cells from cell death induced by the inhibition of topoisomerase I; survival was mediated by an $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin-dependent signal [Uhm et al., 1999].

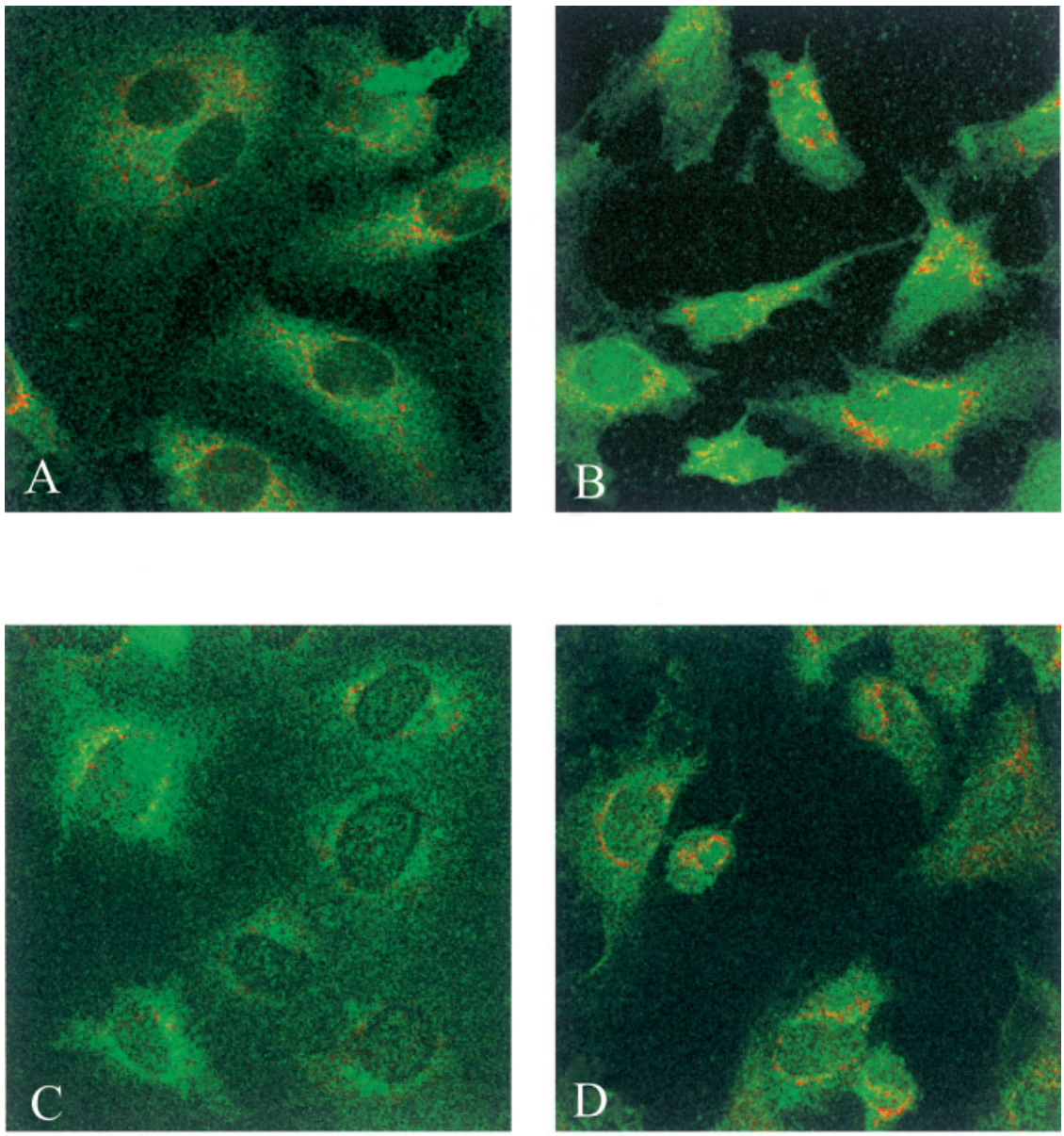


**Fig. 4.** OPN blocks the cellular redistribution Bcl-$X_L$ in the mitochondria of factor-deprived HUVECs. Shown are composite images, obtained by laser scanning confocal microscopy of cells doubly labeled with anti-Bcl-$X_L$ antibody (using a secondary FITC-labeled antibody) and Mitotracker dye. **A**: Control cells in complete medium. **B**: Cells deprived of factors for 12 h. **C** and **D**: Factor-deprived cells after 12 h in medium with 5 and 500 pM bOPN, respectively.

Similar to OPN, this vitronectin-induced survival was accompanied by an increase in the level of Bcl-$X_L$ expression. Integrin-ECM interactions also regulate many developmental and homeostatic processes by supporting cell proliferation, differentiation, and migration. Although not a major component of most ECMs [Hay, 1991; Rittling et al., 2002], OPN is also capable of acting as an ECM molecule by facilitating cell attachment and consequent cell survival [Sauk et al., 1990; Scatena et al., 1998]. Detachment of adherent cells from the ECM leads to cell death by a process that has been termed anoikis [Frisch and Francis, 1994]. Attachment-induced, integrin-mediated activation of focal adhesion kinase is sufficient to suppress anoikis [Frisch et al., 1996], apparently by suppressing Fas-induced apoptosis [Aoudjit and Vuori, 2001], at least in endothelial cells.

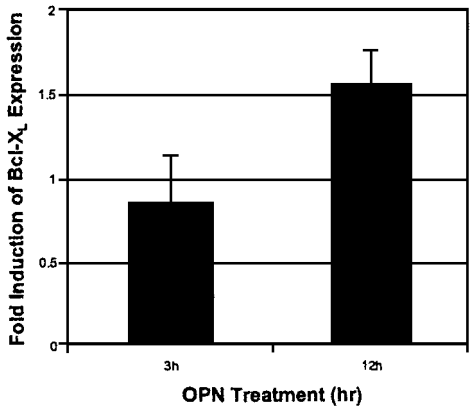


**Fig. 5.** Increase in Bcl-$X_L$ mRNA levels in factor-deprived HUVECs induced by soluble hOPN. The fold-induction by OPN at each time point is the mean of the values obtained from three independent RT-PCR experiments using two independent total cellular RNA samples from treated and control cells. According to Student's *t* test, Bcl-$X_L$ expression increased significantly ($P < 0.05$) between 3 and 12 h of exposure to hOPN.

Survival signals are also provided to adherent cells by soluble cytokines and growth factors. Deprivation of required trophic factors, nerve growth factor for example, results in the apoptosis of sympathetic neurons [Edwards and Tolkovsky, 1994]. Bredesen et al. [1998] have termed receptors capable of initiating an apoptotic signal in the absence of a ligand "dependence receptors," characterized by an intracellular "addiction/dependence domain" that in the absence of ligand favors apoptosis. Thus the neurotrophin receptor $p75^{NTR}$ in the absence of NGF induces a pro-apoptotic state in susceptible cells. Along these same lines, Stupack et al. [2001] have shown that certain unligated integrins behave similarly, inducing apoptosis by activating caspase-8, a process they have termed integrin-mediated cell death. In a related study, Brassard et al. [1999] showed that the $\alpha_v\beta_3$ integrin antagonist echistatin could promote apoptosis of an adherent kidney epithelial cell line (293) engineered to express $\alpha_v\beta_3$ integrin.

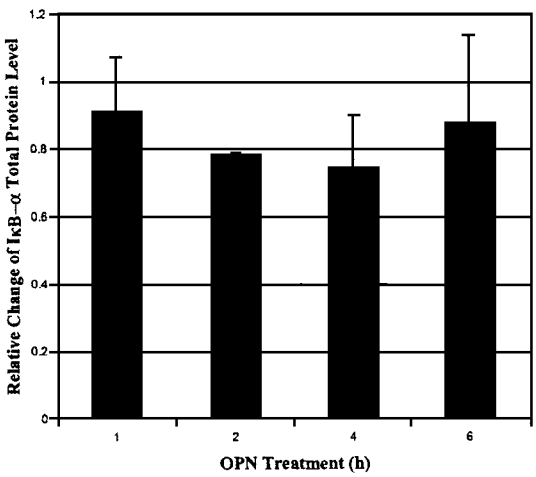


**Fig. 6.** Increased IκB-α degradation in factor-deprived HUVECs in the presence of OPN. IκB-α protein levels were assessed in HUVECs in the presence of bOPN as described in Materials and Methods. For each histogram, the IκB-α total protein level is presented as a ratio of OPN-treated:control cells. Compared to 1-h time point, the level of IκB-α protein level decreased at 2 and 4 h and increased at 6 h. A similar trend was observed in two independent experiments performed under identical conditions.

In the experiments reported above, the HUVECs were adherent to an ECM that they had synthesized on the gelatin-coated plastic during the 4–5 days required for the cells to reach ~80% confluence in complete medium. Substitution of the complete medium with medium lacking required serum and growth factors not only resulted in the cessation of growth but also induced death by apoptosis. Evidently trophic factors in the complete medium were required to maintain cell survival. The thrust of the studies reported here is that soluble OPN can provide the missing trophic signal and maintain cell survival (but not proliferation, data not shown). We believe OPN is not acting as an attachment factor because the cells were already attached to a fully formed matrix that had been laid down previously by the cells in the presence of serum; although HUVECs produce no detectable OPN protein (SAK, unpublished), it is present in the serum, albeit sequestered by Factor H. Recent observations by Lin and Yang-Yen [2001] reveal that OPN can enhance the survival of the non-adherent IL-3-dependent pro-B-cell line Ba/F3 deprived of IL-3; however in this case CD44 was implicated as the critical receptor and PI 3-K/Akt as the critical downstream signal transducing pathway.

Bcl-$X_L$ is normally present at low levels in the cell, sequestered primarily in the mitochondrial membrane in association with other Bcl-2 family members [Desagher and Martinou, 2000; Green, 2000]. It is known to play a direct protective role in HUVECs under normal conditions [Ackermann et al., 1999]. An early response to an apoptotic signal is often the condensation and perinuclear clustering of

mitochondria, likely in response to Bax activation and cytochrome C release. In our experiments (Fig. 5), the redistribution and intensification of the Bcl-$X_L$ label masked whatever changes there might have been in mitochondrial localization. Enhanced expression of Bcl-$X_L$ can inhibit cytochrome C release, Apaf1 activation and cell death in response to an apoptotic signal. Mechanisms underlying this protective action in growth factor-deprived HUVECs may involve heterodimerization with pro-apoptotic members of the Bcl-2 family and the formation of ion channels in the outer mitochondrial membrane that facilitate the ATP/ADP exchange essential for coupling oxidative phosphorylation to cellular respiration and maintenance of mitochondrial integrity [Minn et al., 1999; Vander Heiden et al., 1999; Plas et al., 2001]. Bcl-$X_L$ is also able to suppress κB-mediated gene expression, acting in a negative feedback manner to reduce the pro-apoptotic actions of NF-κB [Grimm et al., 1996].

How might soluble OPN function to enhance cell survival? Denhardt et al. [2001] have proposed that OPN in solution provides a signal that mimics signals provided by the ECM, and thereby enhances survival of non-adherent cells. Although the cells are already adherent in the experimental paradigm used here, OPN is still acting as a trophic factor, engaging unligated integrins and suppressing integrin-mediated cell death. It appears that soluble OPN can stimulate signal transduction pathways similar to those stimulated by matrix-associated OPN [Denhardt et al., 1995; Hruska et al., 1995; Scatena et al., 1998]. Via integrins such as αvβ3, OPN enhances phosphorylation of down-stream mediators of integrin signaling (FAK, Src, paxillin [Denhardt et al., 1995; Hruska et al., 1995]), leading to the activation of NF-κB [Scatena et al., 1998] and consequent stimulation of transcription of target genes such as Bcl-$X_L$. Expression of Bcl-$X_L$ is often increased in response to various survival signals [Grad et al., 2000]. The NF-κB/Rel family has been shown to regulate the apoptotic response by upregulating Bcl-$X_L$ transcription [Chen et al., 2000; Glasgow et al., 2001]. Although, our data do not prove a direct requirement for NF-κB regulation of Bcl-$X_L$ in serum-deprived HUVECs, they do suggest a possible link between the NF-κB signaling pathway and Bcl-$X_L$ function in the context of OPN signaling.

## REFERENCES


Ackermann EJ, Taylor JK, Narayana R, Bennett CF. 1999. The role of antiapoptotic Bcl-2 family members in endothelial apoptosis elucidated with antisense oligonucleotides. J Biol Chem 274:11245–11252.

Adams JM, Cory S. 1998. The Bcl-2 protein family: arbiters of cell survival. Science 281:1322–1326.

Aoudjit F, Vuori K. 2001. Matrix attachment regulates Fas-induced apoptosis in endothelial cells: a role for c-flip and implications for anoikis. J Cell Biol 152:633–643.

Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, Rittling SR, Denhardt DT, Glimcher MJ, Cantor H. 2000. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science 287:860–864.

Asou Y, Rittling SR, Yoshitake H, Tsuji K, Shinomiya K, Nifuji A, Denhardt DT, Noda M. 2001. Osteopontin facilitates angiogenesis, accumulation of osteoclasts, and resorption of ectopic bone. Endocrinology 142: 1325–1332.

Brassard DL, Maxwell E, Malkowski M, Nagabhushan TL, Kumar CC, Armstrong L. 1999. Integrin alpha(v)beta(3)-mediated activation of apoptosis. Exp Cell Res 251:33–45.

Bredesen DE, Ye X, Tasinato A, Sperandio S, Wang JJ, Assa-Munt N, Rabizadeh S. 1998. p75NTR and the concept of cellular dependence: seeing how the other half die. Cell Death Differ 5:365–371.

Chabas D, Baranzini SE, Mitchell D, Bernard CCA, Rittling SR, Denhardt DT, Sobel RA, Lock C, Karpuj M, Pedotti R, Heller R, Oksenberg JR, Steinman L. 2001. The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. Science 294:1731–1735.

Chen C, Edelstein LC, Gelinas C. 2000. The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). Mol Cell Biol 20:2687–2695.

Denhardt DT, Lopez CA, Rollo EA, Hwang SM, An XR, Walther SE. 1995. Osteopontin-induced modifications of cellular functions. In: Osteopontin: role in cell signalling and adhesion. Edited by Denhardt DT, Butler WT, Chambers AF, Senger DR. Ann N Y Acad Sci 760:127–142.

Denhardt DT, Noda M. 1998. Osteopontin expression and function: Role in bone remodeling. J Cell Biochem Suppl 30/31:92–102.

Denhardt DT, Noda M, O'Regan AE, Pavlin D, Berman JS. 2001. Osteopontin—a means to cope with environmental insults: regulation of inflammation, tissue remodeling and cell survival. J Clin Invest 107:1055–1061.

Desagher S, Martinou J-C. 2000. Mitochondria as the central control point of apoptosis. Trends Cell Biol 10: 369–376.

Edwards SN, Tolkovsky AM. 1994. Characterization of apoptosis in cultured rat sympathetic neurons after nerve growth factor withdrawal. J Cell Biol 124:537–546.

Fedarko NS, Fohr B, Robey PG, Young MF, Fisher LW. 2000. Factor H binding to bone sialoprotein and osteopontin enables tumor cell evasion of complement-mediated attack. J Biol Chem 275:16666–16672.

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

Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY. 1996. Control of adhesion-dependent cell survival by focal adhesion kinase. J Cell Biol 134:793–799.

Giachelli CM, Schwartz SM, Liaw L. 1995. Molecular and cellular biology of osteopontin: potential role in cardiovascular disease. Trends Cardiovasc Med 5:88–95.

Glasgow JN, Qiu J, Rassin D, Grafe M, Wood T, Perez-Pol JR. 2001. Transcriptional regulation of the BCL-X gene by NF-kappaB is an element of hypoxic responses in the rat brain. Neurochem Res 26:647–659.

Grad JM, Zeng X-R, Boise LH. 2000. Regulation of Bcl-$X_L$: a little bit of this and a little bit of STAT. Curr Opin Oncol 12:543–549.

Green DR. 2000. Apoptotic pathways: paper wraps stone blunts scissors. Cell 102:1–4.

Grimm S, Bauer MK, Baeuerle PA, Schulze-Osthoff K. 1996. Bcl-2 down-regulates the activity of transcription factor NF-kappaB induced upon apoptosis. J Cell Biol 134:13–23.

Hay ED. 1991. Cell biology of the extracellular matrix. 2nd ed. NY: Plenum Press.

Hruska KA, Rolnick F, Huskey M, Alvarez U, Cheresh D. 1995. Engagement of the osteoclast integrin alpha v beta 3 by osteopontin stimulates phosphatidylinositol 3-hydroxyl kinase activity. Endocrinology 136:2984–2992.

Hwang S-M, Lopez CA, Heck DE, Gardner CR, Laskin DL, Laskin JD, Denhardt DT. 1994. Osteopontin inhibits induction of nitric oxide synthase gene expression by inflammatory mediators in mouse kidney epithelial cells. J Biol Chem 269:711–715.

Ishijima M, Rittling SR, Yamashita T, Tsuji K, Kurosawa H, Nifuji A, Denhardt DT, Noda M. 2001. Enhancement of osteoclastic bone resorption and suppression of osteoblastic bone formation in response to reduced mechanical stress do not occur in the absence of osteopontin. J Exp Med 193:1–7.

Liaw L, Birk DE, Ballas CB, Whitsitt JS, Davidson JM, Hogan BLM. 1998. Altered wound healing in mice lacking a functional osteopontin gene. J Clin Invest 101:1468–1478.

Lin YH, Yang-Yen HF. 2001. The osteopontin-CD44 survival signal involves activation of the phosphatidylinositol 3-kinase/Akt signaling pathway. J Biol Chem 276:46024–46030.

Lopez CA, Carrroll AG, Denhardt DT, Lupu R. 1996. Effect of heregulin on proliferation and expression of apoptosis-related genes: interaction with osteopontin. Proc AACR Ann Mt 37:22.

Mehlen P, Rabizadeh S, Snipas SJ, Assa-Munt N, Salvesen GS, Bredesen DE. 1998. The DCC gene product induces apoptosis by a mechanism requiring receptor proteolysis. Nature 395:801–804.

Minn AJ, Kettlun CS, Liang H, Kelekar A, Vander Heiden MG, Chang BS, Fesik SW, Fill M, Thompson CB. 1999. Bcl-$x_L$ regulates apoptosis by heterodimerization-dependent and heterodimerization-independent mechanisms. EMBO J 18:632–643.

Noiri E, Dickman K, Miller F, Romanoc G, Romanov VL, Shaw R, Chambers AF, Rittling SR, Denhardt DT, Goligorsky MS. 1999. Reduced tolerance to acute renal ischemia in mice with a targeted disruption of the osteopontin gene. Kidney Int 56:74–82.

Oates AJ, Barraclough R, Rudland PS. 1997. The role of osteopontin in tumorigenesis and metastasis. Invasion Metastasis 17:1–15.

Plas DR, Talapatra S, Edinger AL, Rathmell JC, Thompson CB. 2001. Akt and Bcl-$x_L$ promote growth factor-independent survival through distinct effects on mitochondrial physiology. J Biol Chem 276:12041–12048.

Polunovsky VA, Wendt CH, Ingbar DH, Peterson MS, Bitterman PB. 1994. Induction of endothelial cell apoptosis by TNF$\alpha$: modulation by inhibitors of protein synthesis. Exp Cell Res 214:584–594.

Re F, Zanetti A, Sironi M, Polentarutti N, Lanfrancone L, Dejana E, Colotta F. 1994. Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. J Cell Biol 127:537–546.

Rittling SR, Denhardt DT. 1999. Osteopontin function in pathology: lessons from osteopontin-deficient mice. Exp Nephrology 7:103–113.

Rittling SR, Matsumoto HN, McKee MD, Nanci A, An X-R, Novick KE, Kowalski AJ, Noda M, Denhardt DT. 1998. Mice lacking osteopontin show normal development and bone structure but display altered osteoclast formation in vitro. J Bone Miner Res 13:1101–1111.

Rittling SR, Chen Y, Feng F, Wu Y. 2002. Tumor-derived osteopontin is soluble, not matrix associated. J Biol Chem 277:9175–9182.

Sauk JJ, van Kampen CL, Norris K, Moehring J, Foster RA, Somerman MJ. 1990. Persistent spreading of ligament cells on osteopontin/bone sialoprotein-I or collagen enhances tolerance to heat shock. Exp Cell Res 188:105–110.

Scatena M, Almeida M, Chaisson ML, Fausto N, Nicosia RF, Giachelli CM. 1998. NF-$\kappa$B mediates $\alpha_v\beta_3$ integrin-induced endothelial cell survival. J Cell Biol 141:1083–1093.

Schwartz MA. 2001. Integrin signaling revisited. Trends Cell Biol 11:466–470.

Sodek J, Ganss B, McKee MD. 2000. Osteopontin. Crit Rev Oral Biol Med 11:279–303.

Sørensen ES, Petersen TE. 1993. Purification and characterization of three proteins isolated from the proteose peptone fraction of bovine milk. J Dairy Res 60:189–197.

Stupack DG, Puente XS, Boutsaboualoy S, Storgard CM, Cheresh DA. 2001. Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. J Cell Biol 29:155:459–470.

Uhm JH, Dooley NP, Kyritsis AP, Rao JA, Gladson CL. 1999. Vitronectin, a glioma-derived extracellular matrix protein, protects tumor cells from apoptotic death. Clin Cancer Res 5:1587–1594.

Vander Heiden MG, Chandel NS, Schumacker PT, Thompson CB. 1999. Bcl-$x_L$ prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. Mol Cell 3:159–167.

Yoshitake H, Rittling SR, Denhardt DT, Noda M. 1999. Osteopontin-deficient mice are resistant to ovariectomy-induced bone resorption. Proc Natl Acad Sci USA 96: 8156–8160.