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The Antiangiogenic Factor 16K PRL Induces Programmed Cell Death in Endothelial Cells by Caspase Activation

Jean-François Martini, Christophe Piot, Laurent M. Humeau, Ingrid Struman, Joseph A. Martial, and Richard I. Weiner
Center for Reproductive Sciences, Department of Obstetrics, Gynecology and Reproductive Sciences, University of California School of Medicine, San Francisco, California 94143

Abstract
We asked whether the antiangiogenic action of 16K human PRL (hPRL), in addition to blocking mitogen-induced vascular endothelial cell proliferation, involved activation of programmed cell death. Treatment with recombinant 16K hPRL increased DNA fragmentation in cultured bovine brain capillary endothelial (BBE) and human umbilical vein endothelial (HUVE) cells in a time- and dose-dependent fashion, independent of the serum concentration. The activation of apoptosis by 16K hPRL was specific for endothelial cells, and the activity of the peptide could be inhibited by heat denaturation, trypsin digestion, and immunoneutralization, but not by treatment with the endotoxin blocker, polymyxin-B. 16K hPRL-in-duced apoptosis was correlated with the rapid activation of caspases 1 and 3 and was blocked by pharmacological inhibition of caspase activity. Caspase activation was followed by inactivation of two caspase substrates, poly (ADP-ribose) polymerase (PARP) and the inhibitor of caspase-activated de-oxyribonuclease (DNase) (ICAD). Furthermore, 16K hPRL increased the conversion of Bcl-X to its pro-apoptotic form, suggesting that the Bcl-2 protein family may also be involved in 16K hPRL-induced apoptosis. These findings support the hypothesis that the antiangiogenic action of 16K hPRL includes the activation of programmed cell death of vascular endothelial cells. (Molecular Endocrinology 14: 1536-1549, 2000)

INTRODUCTION

Angiogenesis, the formation of new blood vessels, is a necessary component of physiological processes (1) as well as pathological conditions such as tumor growth and metastasis (2, 3). During angiogenesis, capillary endothelial cells proliferate, migrate into tissues, and organize into vessels. Migration and organization of endothelial cells into vessels requires concomitant activation of proteases necessary for tissue remodeling. This cascade of events is under the control of a balance of angiogenic and antiangiogenic factors. Factors known to stimulate the formation of new capillaries *in vivo* (1) include members of the fibroblast growth factors family (FGF) *e.g*. basic FGF (4) and vascular endothelial growth factor (VEGF) (5), angiogenin (6), and angiopoietin-1 (7). Factors with antiangiogenic activity include platelet factor 4 (8), thrombospondin (9), the 16-kDa N-terminal fragment of PRL (16K PRL) (10, 11), angiostatin (12), and en-dostatin (13). More recently, Struman *et al*. (14) demonstrated that recombinant 16-kDa N-terminal fragments of related PRL family members including human GH, GH variant, and placental lactogen also have antiangiogenic activity (14).

The antiangiogenic action of 16K PRL appears to affect the abilities of capillary endothelial cells to proliferate, migrate, and organize into vessels. A high-affinity, saturable, 16K PRL binding site that was independent from the PRL receptor was characterized on capillary endothelial cells (15). We showed that 16K PRL inhibited the proliferative effects of both basic FGF (bFGF) and VEGF on bovine brain capillary endothelial (BBE) cells (10,11). Considerable evidence supports the hypothesis that VEGF stimulates endothelial cell proliferation via activation of the mitogen-acti-vated protein kinase (MAPK) signaling cascade. Binding of VEGF to its receptor (Flk-1/KDR) results in au-tophosphorylation of the receptor, recruitment of the Shc/Grb2/Sos coupling proteins, and activation of Ras and downstream kinases leading to MAPK activation. We demonstrated that 16K human PRL (hPRL) inhibited VEGF-induced activation of MAPK (16). The blockade occurred distal to the activation of the Flk-1 and its association with coupling proteins (17). However, VEGF-induced Ras activation was inhibited by 16K hPRL, which is consistent with the inhibition of the downstream kinases, Raf-1 and MAPK.

16K hPRL was shown to block the organization of BBE cells into polarized capillaries when cultured in type 1 collagen gels (11). *In vivo* tissue remodeling, which accompanies the formation of new vessels, is dependent upon the activation of urokinase (uPA) (18). We showed that 16K hPRL inhibited uPA activity by activation of plasminogen activator inhibitor-type 1 (PAI-1) expression (19).

In addition to the inhibition of endothelial cell proliferation by antiangiogenic factors, there is recent evidence that angiostatin (20) and thrombospondin (21) also activate programmed cell death of endothelial cells. Extensive studies in past few years have led to the identification of many genes regulating programmed cell death during various physiological and pathological processes. Cells undergoing apoptosis are characterized by cytoplasmic shrinkage, membrane blebbing, chromatin condensation, DNA cleavage, and finally, cell fragmentation into membrane-bound apoptotic bodies. Caspase-mediated proteolysis of specific proteins results in this irreversible commitment to cell death (22). At least 13 caspases function as initiators or effectors of the apoptotic signaling pathway (23). Caspases exist as inactive proenzymes, which are activated by cleavage at specific aspartate residues, followed by assembly into heterotetramers. Activation of a caspase can result in stimulation of additional caspases, e.g. autocatalytic activation of caspase 1 by self-aggregation in turn activates caspase 3 (24). Another regulatory component of the apoptotic pathway is the Bcl-2 protein family. These proteins are believed to act at the level of, or upstream to, the caspases to inhibit or facilitate the apoptotic cascade. Antiapoptotic factors include Bcl-2 and Bcl-X_L, while proapoptotic factors include Bax, Bak, Bad, and Bcl-X_s (25).

In the current study, we asked whether 16K hPRL could activate programmed cell death as an additional mechanism for inhibiting angiogenesis. We show that 16K hPRL, but not native 23-kDa PRL, activates DNA fragmentation, and that apoptosis requires activation of the caspase cascade. We demonstrate that the apoptosis-inducing action of the 16K hPRL preparations is dependent on the activity of the peptide and not on endotoxin contamination. Unlike the inhibition of mitogen-induced cell proliferation by 16K hPRL, treatment with 16K hPRL directly activates apoptosis. These findings provide the first detailed and specific analysis of the mechanisms regulating the activation of apoptosis by an antiangiogenic factor.

RESULTS

Stimulation of DNA Fragmentation by 16K hPRL in Vascular Endothelial Cells

The level of DNA fragmentation associated with apoptosis was estimated by measuring the levels of cytosolic mono- and oligonucleosomes with an enzyme-linked immunosorbent assay (ELISA) (25). Experiments were performed in BBE cells deprived of serum [0.5% calf serum (CS)] for 2 days, a treatment that caused a 5-fold increase in nucleosome formation compared with cells cultured in 10% CS (Fig. 1A). Treatment with 2 or 10 nM 16K hPRL for the second day of serum deprivation increased DNA fragmentation 2.3- and 4.4-fold compared with cells cultured in 0.5% CS, respectively. Interestingly, bFGF (0.5 nM) partially reversed the effect of 10 nM 16K hPRL by 50%. To a lesser degree, treatment with VEGF also reduced the effect of 10 nM 16K hPRL by 32%.

The effect of 16K hPRL was similar in other bovine vascular endothelial cells including aortic endothelial (BAE) and the adrenal capillary endothelial (BAC) cells (Fig. 1B and data not shown). Serum deprivation of BAE cells increased DNA fragmentation 2-fold, while 2 nM 16K hPRL caused a further 5-fold increase. Treatment with 0.5 ∩M bFGF had no effect on the serum deprivation-induced effect; however, it reduced the increase by 16K hPRL by 25% (Fig. 1B).

The action of 16K hPRL on DNA fragmentation was dose dependent. Increasing concentrations of 16K hPRL from 0.1 to 25 nM caused an exponential increase in DNA fragmentation in BBE cells (Fig. 1C). The highest concentration of 16K hPRL caused a 14-fold increase in DNA fragmentation as compared with 0.5% CS. The first significant increase in DNA fragmentation (2-fold) after treatment with 2 nM 16K hPRL was detectable at 1 h in floating cells and at 2 h in attached cells (Fig. 1D). 16K hPRL-induced DNA fragmentation continued to increase for up to 24 h. The time course of the response in floating and attached cells was parallel for 8 h, but was then divergent, as it increased linearly in attached cells and plateaued in floating cells.

To distinguish the effect of 16K hPRL on apoptosis from that of serum deprivation, we studied the effect of 16K hPRL on DNA fragmentation in different concentrations of serum in BBE cells. Increasing serum concentration decreased the amplitude of 16K hPRL-induced DNA fragmentation (Fig. 1E). As previously observed in BBE cells cultured in 0.5% CS, 2 and 10 nM 16K hPRL caused a 2- and 4-fold increase in DNA fragmentation, respectively. In 1% and 5% CS, treatment with 10 nM 16K hPRL induced a 4- and 10-fold increase in DNA fragmentation compared with the untreated control, respectively. Finally, in 10% CS, 2 and 10 nM 16K hPRL caused a 3- and 8-fold increase in DNA fragmentation, respectively (Fig. 1E). These results show that the stimulation of DNA fragmentation by 16K hPRL is not dependent on serum concentration. In human umbilical

vein endothelial (HUVE) cells, similar results were obtained. In 10% FCS, 1 and 10 nM 16K hPRL caused a 3.2- and 6.5-fold increase in DNA fragmentation, respectively (Fig. 1F). Interestingly, tumor necrosis factor-α (TNFα) (5 ng/ml) had no significant effect on nucleosome formation in 10% FCS, while it caused a 7.1-fold increase under serum-starved conditions. Finally, endotoxin standards-1 [E-Toxate kit, Sigma, St. Louis, MO; 10 endotoxin units (EU)/ml] and -2 (Re 595, Sigma, 5 ng/ml) induced a 3.5 ± 0.2 and 4.4 ± 0.3 fold increase DNA fragmentation, respectively, which were significantly less than the 6.5 ± 0.3 fold increase due to 10 nM 16K hPRL ($n = 5$, $P < 0.05$).

Specificity of 16K hPRL Preparation

Since the 16K hPRL was produced in *Escherichia coli* it was important to demonstrate that the activity of the preparation used was not caused by a contaminant, *e.g.* endotoxin. A well defined property of endotoxin [or lipopolysaccharide (LPS)] in many cultured cells is the induction of programmed cell death (26, 27). In BBE cells, while 0.5 EU/ml endotoxin-1 had no effect (Fig. 1A), the first detectable increase in DNA fragmentation was observed at a concentration of 1 EU/ml of endotoxin-1, which caused a significant 2-fold increase *vs.* 0.5% CS control ($P < 0.05$) (data not shown). Treatment with 10 nM 16K hPRL increased DNA fragmentation 5.7-fold, while treatment with 10 EU/ml endotoxin-1 caused a similar 6.1-fold increase compared with culture in 0.5% CS (Fig. 2). This is 1300 times the amount of endotoxin present in 320 ng of 16K hPRL preparation used throughout the study (amount present in 2 ml of a 10 nM solution).

Heat denaturation of 16K hPRL by boiling the sample for 2 min before adding it to the cells completely inhibited the 16K hPRL stimulation of DNA fragmentation. Similarly, the complete digestion of 16K hPRL by treatment with trypsin abolished its activity (Fig. 2). Neither treatment had any effect on the action of endotoxin-1. Furthermore, pretreatment with 10 μg/ml (final concentration) of the endotoxin blocker, poly-myxin-B, completely abolished the endotoxin effect, but had no effect on the action of 16K hPRL.

To further dissociate the action of 16K hPRL from endotoxin, we showed that 16K hPRL caused a 4.8-fold increase in DNA fragmentation over serum deprivation in HUVE cells, a response similar to that seen in BBE cells. In comparison 5 ng/ml of TNFα caused a similar 4.8-fold increase in DNA fragmentation, while 10 EU/ml of endotoxin-1 caused a 3.2-fold increase. However, in primary endometrial stromal cells and in endometrial epithelial (Ishikawa) cells, while 10 nM 61K hPRL had no effect on DNA fragmentation, 5 ng/ml of TNFα and 10 EU/ml of endotoxin-1 stimulated DNA fragmentation in both cell types (Fig. 3, B and C).

Immunoneutralization of 16K hPRL was able to block its ability to induce DNA fragmentation in BBE cells (Fig. 4A). Both a 1:500 and 1:1000 dilution of monoclonal antibodies 4G7 (16K hPRL specific) or 6E4 (recognizes both 16K hPRL and intact 23-kDa hPRL) inhibited 16K hPRL activity. However, preincubation of 16K hPRL with a 1:500 dilution of an unrelated monoclonal antibody (Ctrl) had no effect on the stimulation of DNA fragmentation. Monoclonal antibodies 6E4 and 4G7 (1:500 dilution) also abolished 16K hPRL activity in HUVE cells, but had no effect on TNFα and endotoxin-2 activity (Fig. 4B). Finally, a neutralizing antibody against human TNFα was able to block the TNFα effect, but neither the 16K hPRL nor the endotoxin-2 effects. Preincubation with the unrelated antibody had no effect on any response.

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Fig. 1. *16K hPRL Induces DNA Fragmentation in Bovine Endothelial Cells.*

A, BBE cells were cultured in 0.5% CS or 10% CS for 24h. For a second 24 h cells were cultured in 0.5% or 10% CS, or were treated with 0.5 nM bFGF, 2 nM VEGF, 2 or 10 nM 16K hPRL, or 16K hPRL in combination with the mitogens in 0.5% CS. If not mentioned in the figure legend, this design was used throughout the studies. Endotoxin-1 (0.5 EU/ml) was added as a control for endotoxin contamination in the 16KhPRL preparation. DNA fragmentation was measured using an ELISA assay and is expressed as an enrichment factor (ratio of the absorbance of the sample to that measured for 10% CS). Each bar represents the mean ± SD, n = 6. B, Similar experiments were performed in bovine aortic endothelial (BAE) cells. The caspase inhibitor, z-VAD-fmk (20 μM), was added to determine whether the ability of 16K hPRL to stimulate DNA fragmentation in BAE cells depended on caspase activation. Each bar represents the mean ± SD, n = 4. C, Dose-dependent stimulation of DNA fragmentation by increasing concentrations of 16K hPRL in BBE cells. Each bar represents the mean ± SD, n = 3. The *asterisk* denotes a *P* value of < 0.05 vs. 0.5% CS control. D, Kinetics of 16K hPRL-induced BBE cell DNA fragmentation. Cells were cultured in 0.5% CS for 24 h and subsequently treated or not (*open square* and *circle*) with 2 nM 16K hPRL for the indicated time (*filled* square and circle). DNA fragmentation was measured in cells attached to the plastic dish (*square*) or in cells floating in the conditioned medium (circle). Each point represents the mean ± SD, n = 3. (E and F) To test whether the stimulation of DNA fragmentation by 16K hPRL was dependent on serum concentration, BBE cells were cultured in 0.5, 1, 5, or 10% CS for 48 h, while HUVE cells were cultured in 0% or 10% serum. E, Cells were treated with nothing or 2, or 10 nM 16K hPRL for the last 24 h. F, Cells were treated with nothing, 1 or 10 nM 16K hPRL, 5 ng/ml TNFα, 10 EU/ml endotoxin-1, or 5 ng/ml endotoxin-2. Each *bar* represents the mean ± SD, n = 5. The *asterisk* denotes a *P* value of < 0.05 *vs.* the nonstimulated control.

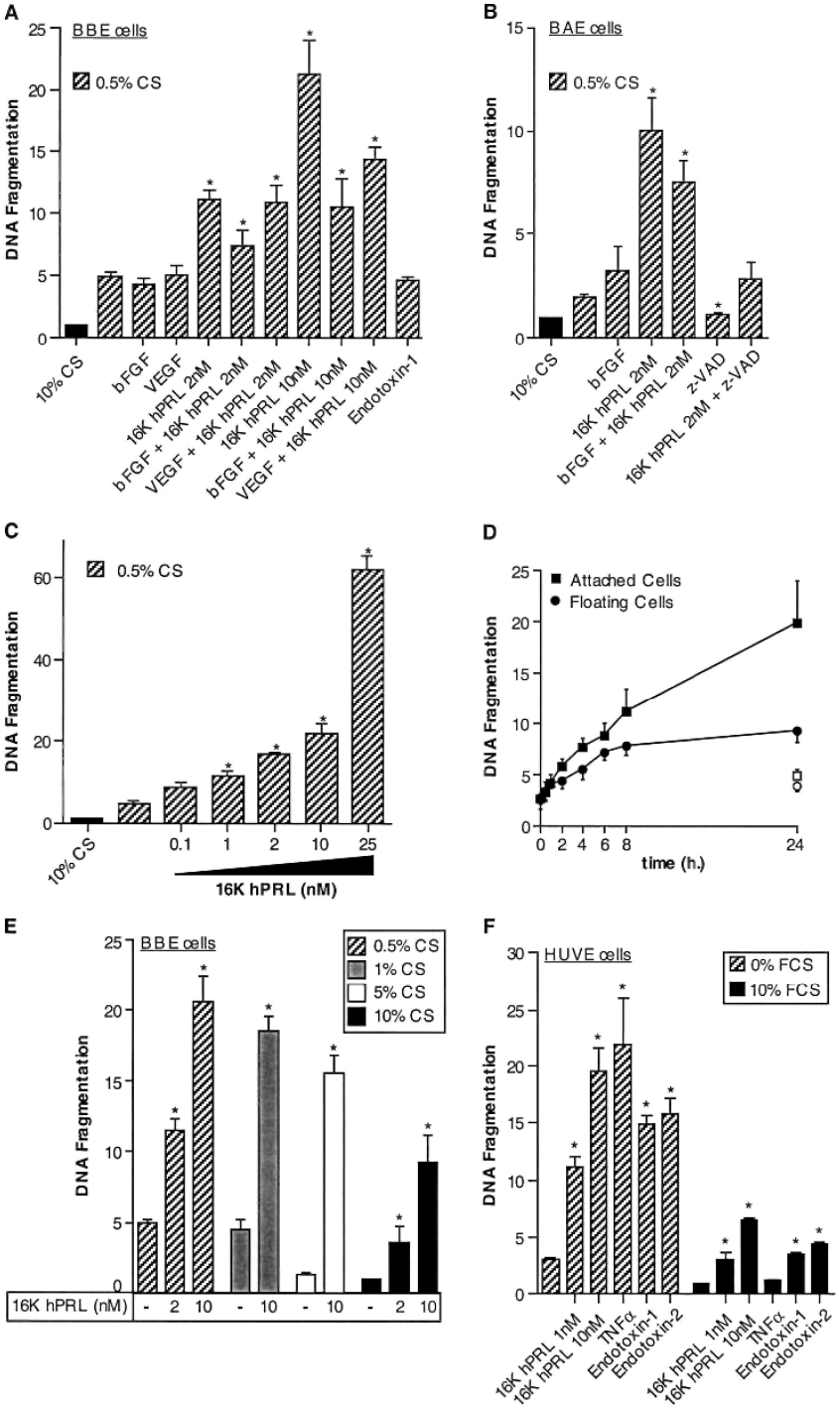

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Fig. 2. *Stimulation of DNA Fragmentation by 16K hPRL Is Not Dependent on Endotoxin Contamination.* BBE cells were cultured in 0.5% or 10% CS and treated for 24 h with 10 nM 16K hPRL or 10 EU/ml endotoxin-1. 16K hPRL and endotoxin samples were also pretreated with Poly-myxin-B, boiling for 2 min, and trypsin digestion before being added to the cells. Each bar represents the mean ± SD, n = 3. The *asterisk* denotes a *P* value of < 0.05 vs. the corresponding 0.5% CS control.

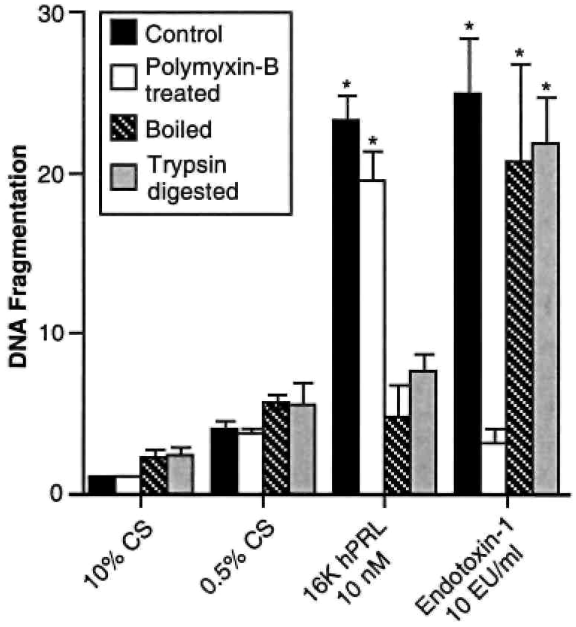
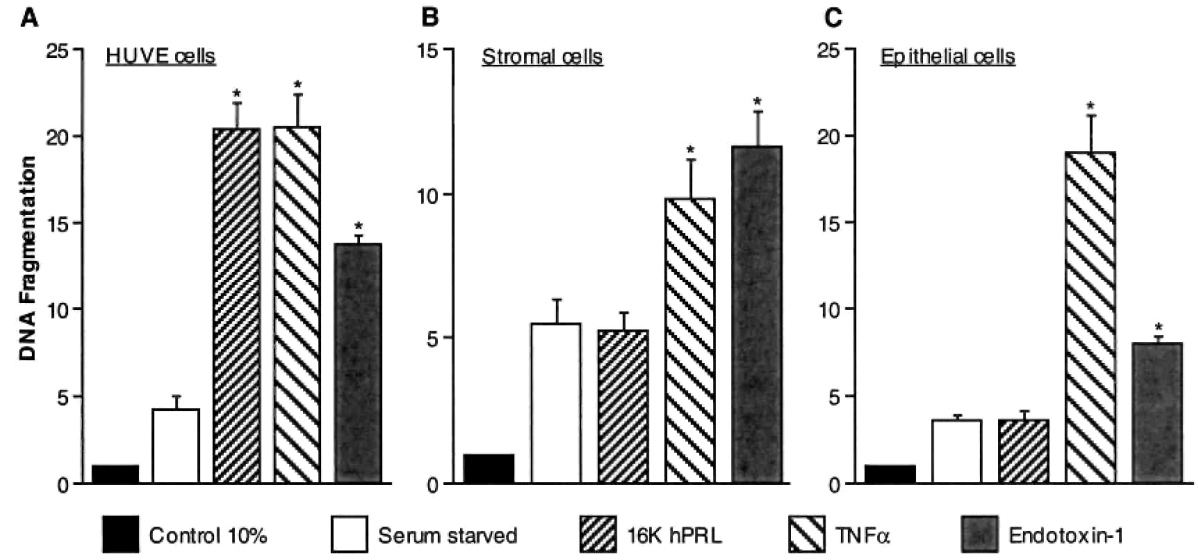


Fig. 3. *16K hPRL Induces DNA Fragmentation Specifically in Endothelial Cells.* HUVE (A), human primary stromal endometrial (B), and human epithelial endometrial (C) cells were cultures in 10% serum or serum starved and treated for 24 h with either 10 nM 16K hPRL, 5 ng/ml TNFα, or 10 EU/ml endotoxin-1. Each *bar* represents the mean ± SD, n = 4. The asterisk denotes a P value of < 0.05 vs. the serum-starved control.



These results demonstrate that the ability of the 16K hPRL preparations to stimulate DNA fragmentation was dependent on the activity of the peptide, and not on endotoxin or contaminating proteins in the preparation. The results also show that the action of 16K hPRL is specific for vascular endothelial cells. Furthermore, the experiments in HUVE cells show that the action of 16K hPRL is not mediated by the stimulation of the release of TNFα.

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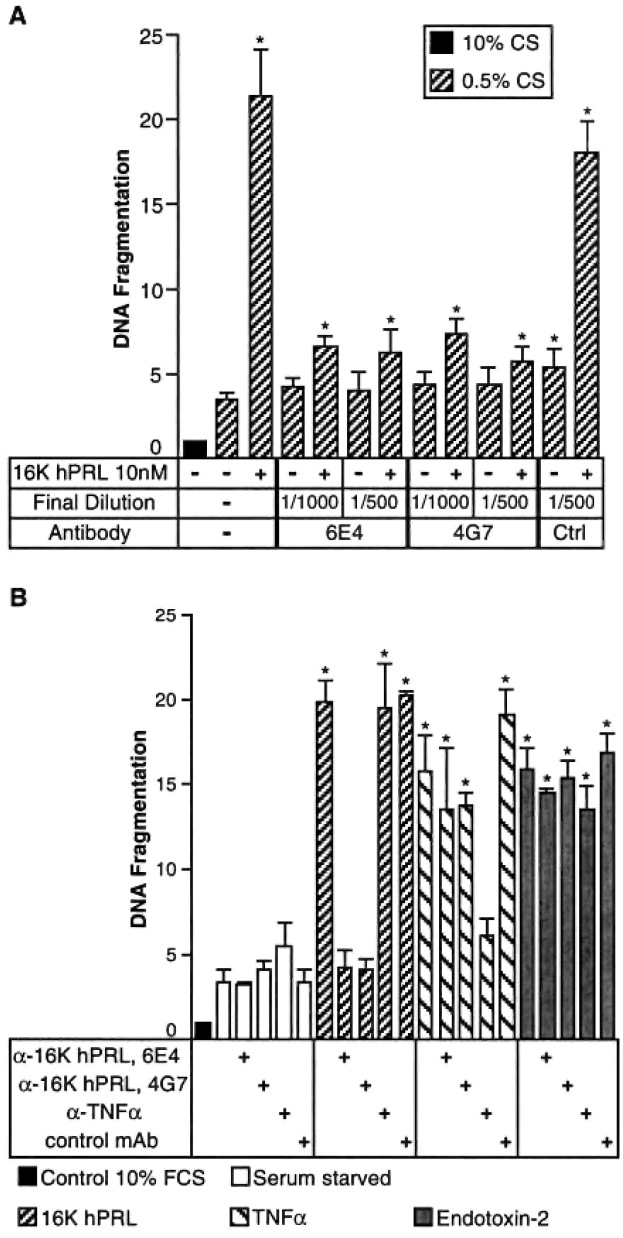
***Fig. 4**. Immunoneutralization of 16K hPRL Effect.*
ABBE cells were cultured in 0.5% or 10% CS and treated for 24 h. Samples containing 10 nM 16K hPRL were preincu-bated with either a monoclonal antibody that recognizes both native 23 kDa hPRL and 16K hPRL (6E4), a monoclonal antibody that is specific for 16K hPRL (4G7), or a control monoclonal antibody that is unrelated to 16K hPRL (Ctrl). B, HUVE cells were cultured in 10% serum or serum starved and treated for 24 h with 10 nM 16K hPRL, 5 ng/ml TNFα, or 5 ng/ml endotoxin-2. Indicated samples were preincubated with the monoclonal antibodies 6E4 or 4G7, or an immuno-neutralizing antibody specific for human TNFα, or the control monoclonal antibody (control mAb). Each bar represents the mean ± SD, n = 3. The asterisk denotes a P value of < 0.05 vs. the corresponding serum-starved control.



Action of 16K hPRL Is Not Mediated via PRL or GH Receptors

To demonstrate that 16K hPRL is not interacting with the GH or PRL receptor family to transduce its signal, we tested the effect of hGH and hPRL alone or in combination with 16K hPRL (Fig. 5). Addition of 10 and 50 nM of either recombinant hGH or hPRL alone after serum deprivation of BBE cells had no significant effects on DNA fragmentation. Once again these results argue against the possibility that the action of 16K hPRL is due to endotoxin contamination, since both recombinant hPRL and hGH had no effect on DNA fragmentation even though the concentration used had equivalent or higher endotoxin content than the 16K hPRL preparation. Furthermore, addition of 25 times more hGH or hPRL (50 n M) had no antagonistic effect on the ability of 2 nM 16K hPRL to stimulate DNA fragmentation (Fig. 5). The results suggested that, as for inhibition of mitogen-induced cell proliferation (11) or activation of PAI-1 expression (19), the 16K hPRL effects are not mediated by the PRL or GH receptors, but rather by a yet unknown type of receptor.

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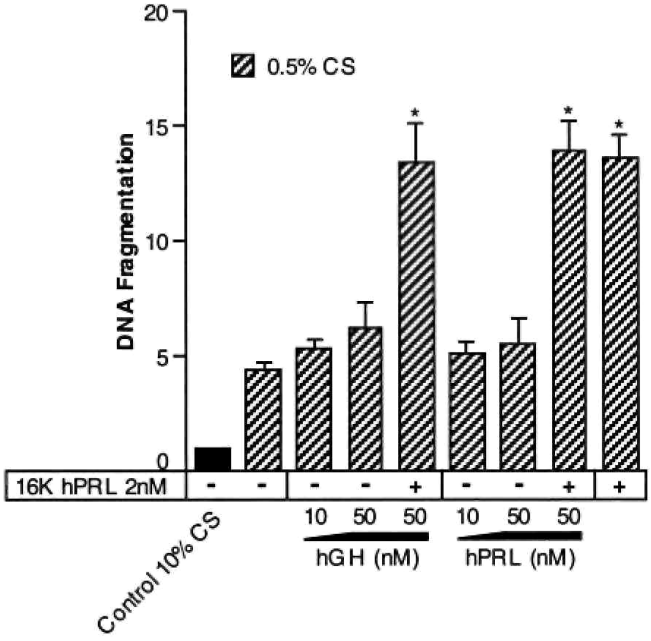
Effect of 16K hPRL on DNA Cell Content

To directly investigate the effect of 16K hPRL on the number of cells entering programmed cell death, we analyzed the DNA content of 16K hPRL-treated BBE cells using flow cytometry of propidium iodide-stained cells. After 2 days of serum deprivation, BBE cells were relatively well synchronized in G_0/G_1 phase (Fig. 6A, *top panel*), with 17% of the cells having reduced DNA content (undergoing programmed cell death). After 24 h of 16K hPRL treatment (2 nM), more than 50% of the cells were undergoing apoptosis. Treatment with 16K hPRL drastically diminished the population of cells in G_2/M phase, as well as the population of cells in G_0/G_1, phase (Fig. 6A, *bottom panel*). Kinetically, we observed the same pattern of changes in DNA content after 16K hPRL treatment as had been observed for DNA fragmentation (Fig. 6B). The dying cell population doubled after 1 h of treatment and was still increasing at 24 h. These results indicate that 16K hPRL not only increased the DNA fragmentation in cells already undergoing apoptosis after serum deprivation, but also recruited new cells into this pathway.

Similar effects on DNA content were observed when experiments were performed in 10% CS. Cells cultured in 10% CS showed three distinct populations, 52% of the cell being in G_0/G_1, phase, 38% being in S or G_2/M phases, and only 10% of the cells undergoing programmed cell death (Fig. 6C, *top panel*). After 24 h treatment with 2 nM 16K hPRL, the population of cells undergoing mitosis was dramatically diminished, to 7%, as was the population of cells in G_0/G_1 phase (24%). A similar percentage of cells (68%) were undergoing apoptosis in 10% CS as seen with 0.5% CS (50%) (Fig. 6C, *bottom panel*). Kinetically, we observed the same dynamics of the changes in DNA content after 16K hPRL treatment as seen for cells cultured in 0.5% CS. The apoptotic cell population doubled after 1 h and was still growing between 8 and 24 h (Fig. 6D).

Fig. 5*. Effect of 16K hPRL Is Not Mediated by the GH or PRL Receptors*
BBE cells were cultured in normal 10% CS conditions, and then serum deprived or not for 24 h, and finally treated for another 24 h with increasing concentrations of hGH or hPRL alone, with 2 nM 16K hPRL alone, or in combination with 50 nM hGH or hPRL to test the agonistic or antagonistic effects of 16K hPRL to hPRL and hGH. Each bar represents the mean ± SD, n = 3. The asterisk denotes a P value of < 0.05 vs. 0.5% CS control.



16K hPRL Activates the Caspase Cascade

We then asked whether the stimulation of apoptosis by 16 K hPRL was associated with activation of the caspase cascade. By Western blotting the 32-kDa proform of caspase 3 was expressed in BBE cells before and after serum deprivation (Fig. 7A). The 17-kDa active subunit of caspase 3 was not present in cells cultured in 10% CS, while after culture in 0.5% CS for 24 h a faint 17-kDa band corresponding to the active subunit of caspase 3 began to appear. Treatment with 16K hPRL alone or in combination with bFGF induced a large increase in the

formation of the active 17-kDa form, while the amount of the 32-kDa proform was decreased (treatment with 16K hPRL in combination with bFGF) or unchanged (treatment with 16K hPRL alone).

To confirm that the increase in the 17-kDa band was associated with an increase in caspase 3 activity, we analyzed one of the known substrates of caspase 3, the poly(ADP-ribose) polymerase (PARP). This 116-kDa DNA repair enzyme is cleaved to an inactive 85-kDa fragment by caspase 3 (Fig. 7B, *upper panel*). By densitometric analysis, treatment with 16K hPRL resulted in the conversion of 65% of PARP into the inactive 85-kDa fragment (Fig. 7B, *lower panel*). Serum deprivation only resulted in the conversion of 15% of the PARP, while at the basal level, in 10% CS, the conversion level was 5%. Treatment with bFGF was not able to significantly reverse the effect of 16K hPRL. We then asked whether treatment with 16K hPRL activated caspase 1, a potential upstream step in the caspase cascade. Caspase 1 has been shown to activate caspase 3 in response to TNFα (28), as well as during photooxidative stress (29). By Western blotting, caspase 1 was present in BBE cells as the inactive 45-kDa proenzyme (Fig. 7C). Treatment with 16K hPRL, at two different doses (2 and 10 nM), or in combination with bFGF, stimulated the formation of the 20-kDa fragment of caspase 1, known to be part of the active protease complex. This band was absent before serum deprivation and in most of the experiments after serum deprivation, or after treatment with bFGF or native hPRL. The effect of 10 nM 16K hPRL on caspase 1activation was independent of serum concentration, since it was observed in 1, 5, and 10% CS (Fig. 7D). Treatment with 1 nM 16K hPRL was capable of inducing the formation of the 20-kDa fragment in 10% CS, while serum deprivation resulted in the appearance of only a faint band (Fig. 7D). Endotoxin-1 (1 EU/ml) was not able to induce the processing of caspase 1 to its active form (Fig. 7C). The effect of 16K hPRL was inhibited by treatment with the general caspase inhibitor, z-VAD (30). A similar effect of z-VAD treatment was observed in BAE cells. The increase in DNA fragmentation induced by 2 nM 16K hPRL in 0.5% CS was abolished by pretreatment of the cells with 20 μM z-VAD (Fig. 1B). These results were consistent with the idea that 16K hPRL specifically activated an apo-ptotic cascade involving the activation of caspase 1.

The kinetics of caspases 1 and 3 processing were similar under 0.5 and 10% CS culture conditions (Fig. 7E, 0.5% CS and 10% CS). The active forms of the proteases were observed as early as 15 min after treatment with 2 nM 16K hPRL. By 30 min, activation of caspase 3 resulted in the cleavage of PARP (116 kDa), resulting in the appearance of the inactive 85-kDa form. The 85-kDa cleaved form was observed throughout the 24-h treatment, while the intact PARP could no longer be observed after 3 h in 10% CS and was almost undetectable in 0.5% CS. The inhibitor of the caspase-activated DNase (ICAD) (31, 32), another potential substrate of caspase 3, was cleaved to its inactive 34-kDa form in a similar pattern by treatment with 16K hPRL. This suggests that 16K hPRL specifically activated an apoptotic cascade involving the activation of caspases, like caspase 1 or 3, and subsequently the inactivation of downstream substrates like PARP or ICAD.

Effects of 16K hPRL on Expression of the Bcl-2 Family

Among the large Bcl-2 protein family, we analyzed the expression of one antiapoptotic protein, Bcl-2, and two proapoptotic proteins, Bax and Bak, by Western blotting (33). After serum deprivation, the amount of Bcl-2 was markedly increased (Fig. 8A). Treatment of the BBE cells with 2 or 10 nM 16K hPRL did not significantly modify this increased expression. The levels of Bax and Bak were unchanged after culture in 0.5% CS or treatment with 2 or 10 nM 16K hPRL. Densitometric analysis and expression of the results as the Bcl-2/Bax ratio also showed no changes (Fig. 8B).

Similarly, in kinetic studies, treatment with 2 nM 16K hPRL had little effect on the level of Bcl-2, Bax, or Bak over a 24-h period (Fig. 8C, 0.5% CS and 10% CS). However, treatment with 16K hPRL stimulated the conversion of Bcl-X from its antiapoptotic 29-kDa form (Bcl-X_L) to its proapoptotic 21-kDa form (Bcl-X_s) (34). Clear increases in Bcl-X_s were observed 1 h after treatment with 16K hPRL, and at 3 h, 50 and 20% of Bcl-X_L was converted to Bcl-X_s in 0.5% CS and 10% CS, respectively. These results suggest that 16K hPRL had no major effect on Bcl-2, Bax, and Bak levels. However, stimulation of the conversion of Bcl-X_L to inactive Bcl-X_s by 16K hPRL is consistent with the activation of apoptosis. This observation may help to explain why the increase in Bcl-2 expression observed after culture in 0.5% CS was not sufficient to prevent the apoptotic effect of 16K hPRL

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Fig. 6. *Stimulation of Apoptosis by 16K hPRL as Determined by DNA Content Analysis by Flow Cytometry.*
BBE cells were cultured in 0.5% CS (A) or 10% CS (C) for 24 h and then treated with nothing (Control) or 2 nM 16K hPRLfor 24 h. Events falling under the 100 (0 to 100) FL2 channel scale are considered as apoptotic events resulting from DNA fragmentation. Percents represent the proportion of gated cells undergoing apoptosis. Position of the G_0/G_1, S, G_2/M, and apoptosis phase is indicated on top of the right panel (A). B and D, Percentage of cells undergoing apoptosis after treatment with 2 nM 16K hPRL with time. Each point represents the mean value of two separate experiments.

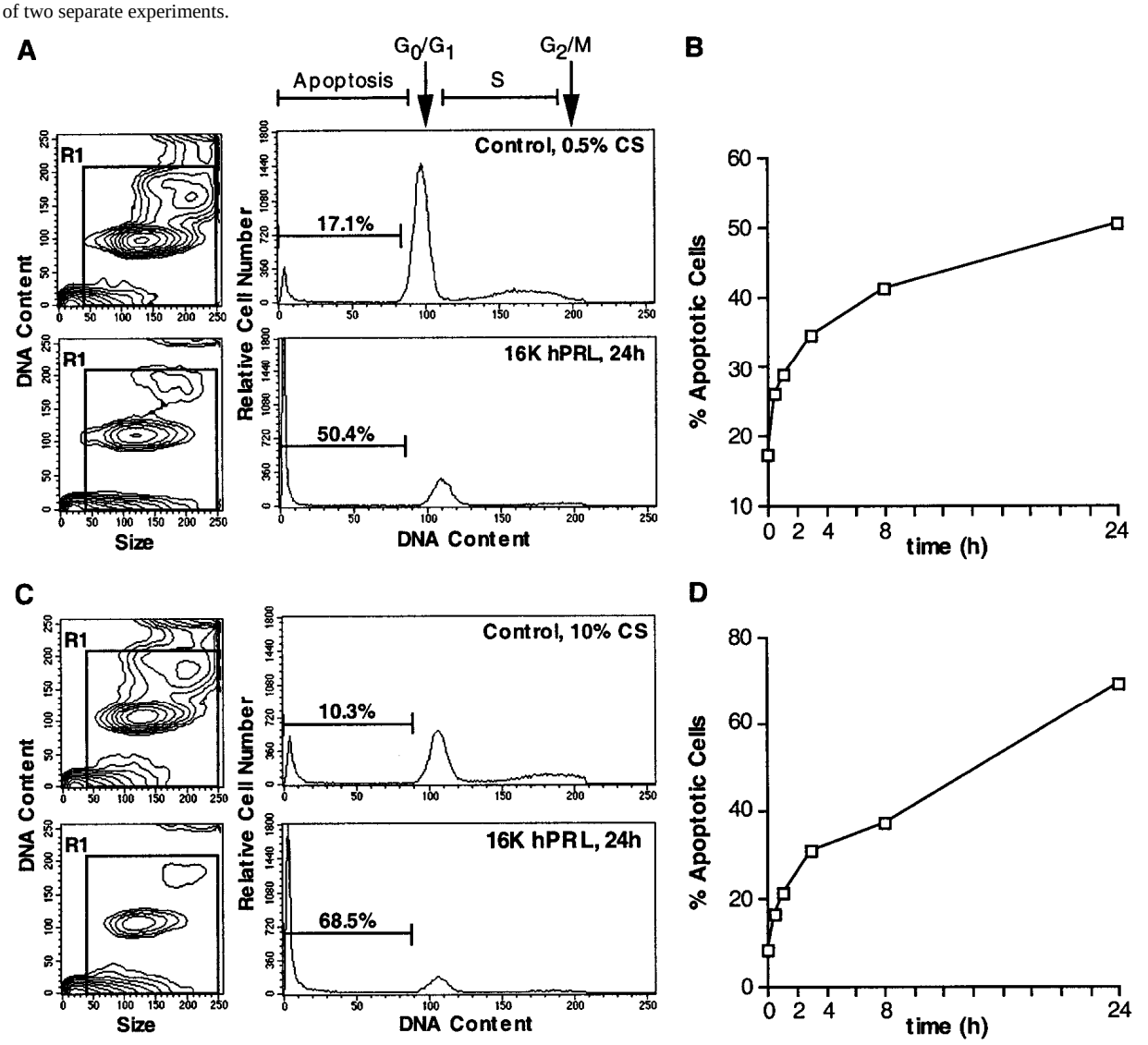

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Fig. 7. *Activation of Caspases 1 and 3 by 16K hPRL*

A, Western blot analysis of caspase 3 in lysates of BBE cells treated for 24 h with 0.5 nM bFGF, 2 nM 16K hPRL, or both in 0.5% CS. The molecular masses of the inactive proform (32 kDa), partially processed inactive form (28 kDa), and active subunit (17 kDa) of caspase 3 are indicated on the left side. B, upper panel: Western blot analysis using an anti-PARP antibody (PARP). The active 116 kDa form of PARP was converted to the inactive 85 kDa form after activation of caspase 3. B, bottom panel: The mean ± SD (n = 4) relative intensity of the 116 and 85 kDa bands determined by ImageQuant Software (Molecular Dynamics, Inc.). The asterisk denotes a P value of < 0.05 vs. 0.5% CS control. C, Western blot analysis of the inactive 45 kDa proform, partially processed inactive 30 kDa form, and 20 kDa active form of caspase 1, after treatment with 2 or 10 nM 16K hPRL, 0.5 nM bFGF, and zVAD, alone or in combination. The effects of treatment with 23K hPRL or endotoxin-1 were also tested. D, Effect of CS concentration (1%, 5%, 10%, or 0.5%) on the activation of caspase 1 by 16K hPRL. E, Time course of the activation of caspases 1 and 3 and inactivation of caspase 3 substrates, PARP and ICAD, after treatment with 2 nM 16K hPRL in 0.5% or 10% CS. Sizes of the expected proteins are indicated in between the two panels.

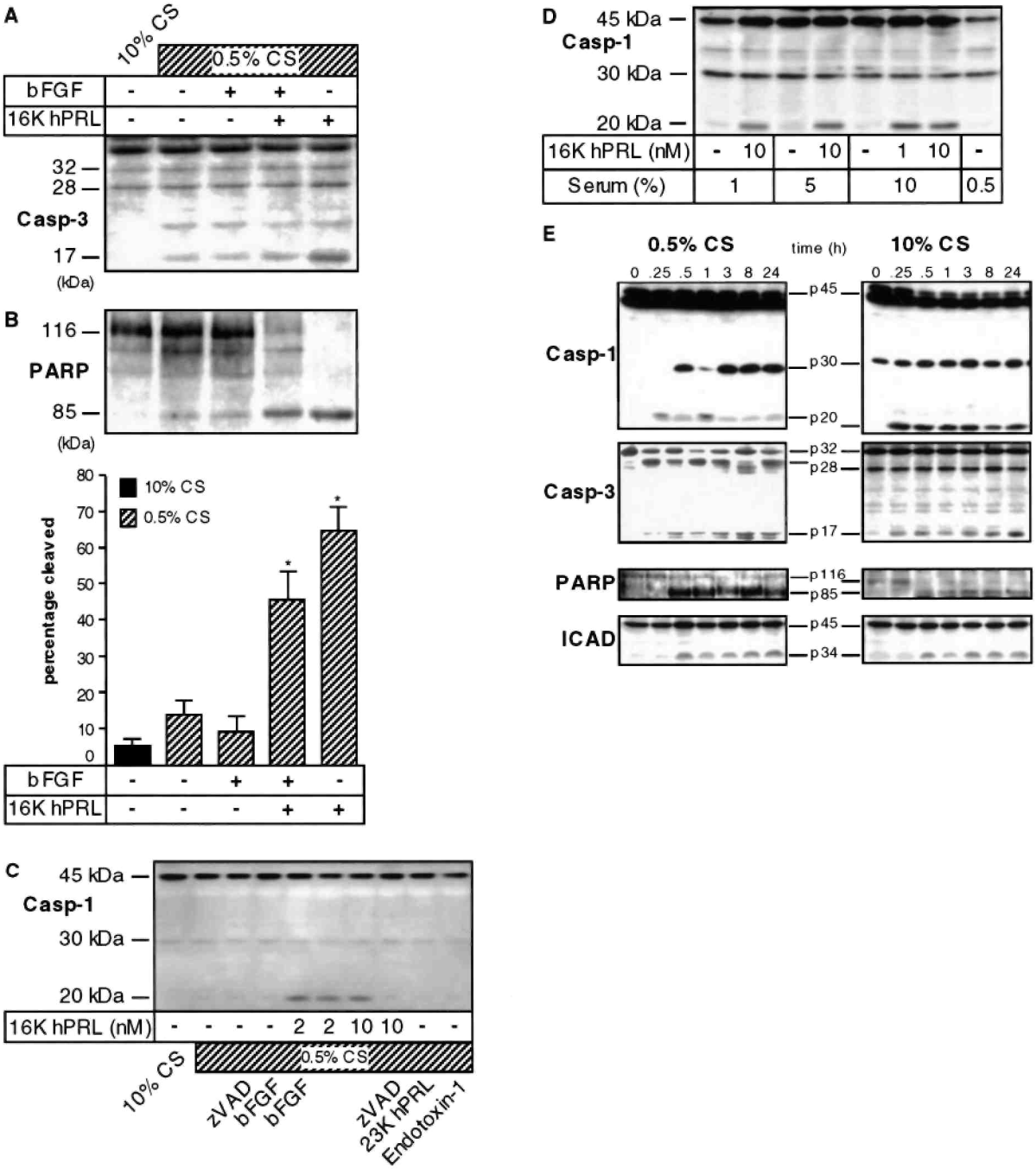

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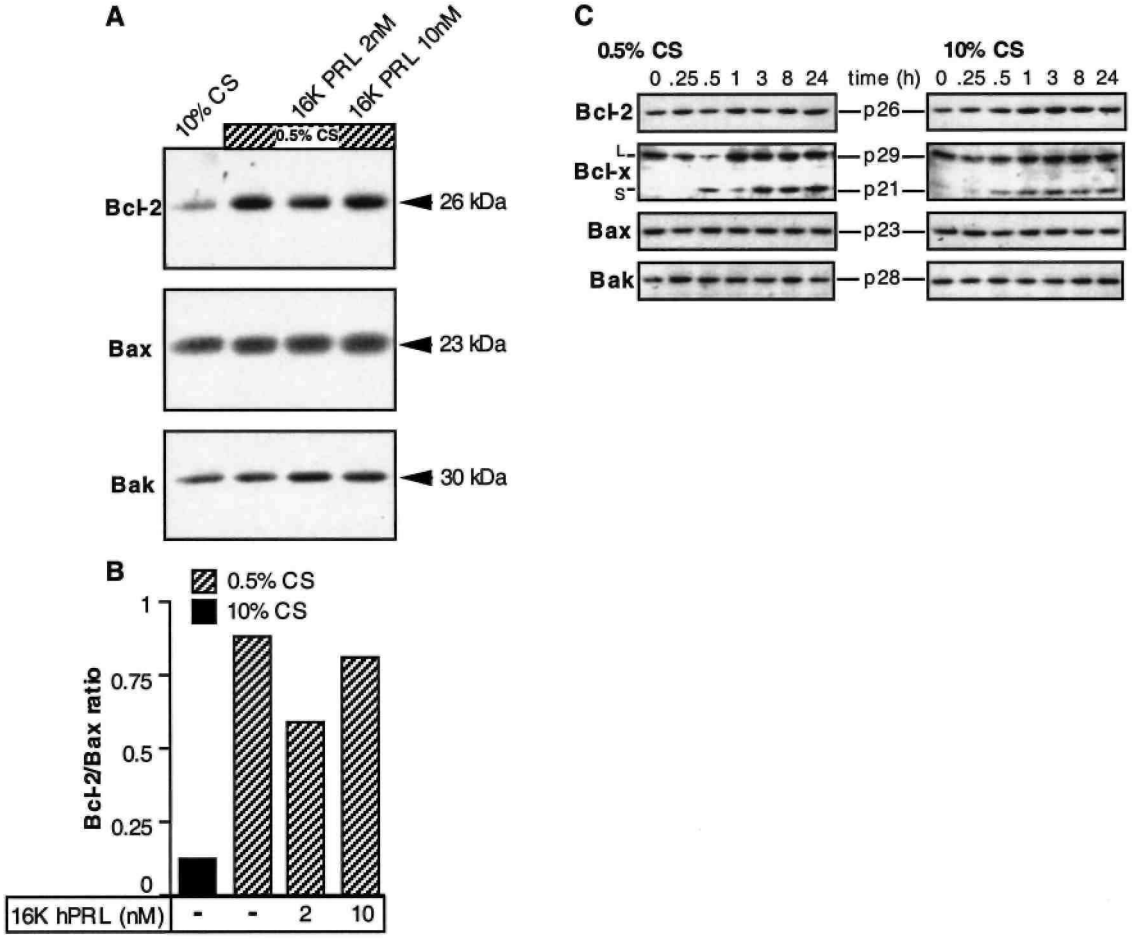
Fig. 8. *Effect of Treatment with 16K hPRL on Levels of Bcl-2, Bax, Bak, and Bcl-X*
A, Western blot analysis of Bcl-2, Bax, and Bak after treatment with 2 or 10 nM 16K hPRL in 0.5% CS as compared with culture in 0.5 or 10% CS. Size of the corresponding proteins was indicated on the right side. B, The relative intensity of 26 and 23 kDa bands, corresponding to Bcl-2 and Bax, respectively, and the mean (n = 2) Bcl-2/Bax ratio calculated from the intensity of the bands determined by the ImageQuant Software (Molecular Dynamics, Inc.). C, Time course of the effect of treatment with 2 nM 16K hPRL on the level of Bcl-2, Bcl-X, Bax, and Bak as analyzed by Western blot in 0.5 and 10% CS. The 29 kDa antiapoptotic form of Bcl-X (Bcl-X_L) was converted to the 21 kDa proapoptotic form (Bcl-X_s) by treatment with 16K hPRL Sizes of the expected proteins are indicated in between the two panels.



DISCUSSION

These findings demonstrate that 16K hPRL specifically stimulates programmed cell death of vascular endothelial cells by multiple criteria: DNA fragmentation; fluorescence-activated cell sorting (FACS) analysis of BBE cell DNA content; activation of the caspase cascade; and regulation of the Bcl-2 family members. In addition, treatment with 16K hPRL inhibited entry of BBE cells into the cell cycle at the G_0/G_1, transition. After 24 h of 16K hPRL treatment, more than 50% of the cells were undergoing apoptosis, while the reminder of cells were mainly in the G_0/G_1 state. These data support the earlier observations that 16K hPRL inhibits FGF-induced BBE cell proliferation, which was followed by rounding up of the cells (11); they show in addition that 16K hPRL stimulates apoptosis. This induction of programmed cell death was dependent on the caspase cascade activation, as well as the inacti-vation of Bcl-X.

Bacterial endotoxin (LPS) has been shown to increase PAI-1 expression, stimulate apoptosis, and inhibit cell proliferation of endothelial cells (35, 36). The observation regarding the similarity of the signaling pathways activated by LPS and 16K hPRL made it imperative to demonstrate that the antiangiogenic actions of the recombinant 16K hPRL preparations was not due to contamination with endotoxin. First the endotoxin level of the 16K hPRL preparation used in this study was more than a 1,000-fold less than necessary to observe any biological effect. The ability of the 16K hPRL preparations to stimulate DNA fragmentation could be completely blocked by digestion of the protein with trypsin, or denaturation of the protein by boiling. The action of LPS was unaffected by boiling but was blocked by the addition of polymyxin-B. However, polymyxin-B treatment had no effect on the action of 16K hPRL. That the activity of the 16K hPRL could depend on complexing with LPS in

the preparations appears unlikely, since for every 3,000 16K hPRL molecules, there is approximately 1 LPS molecule. Human endothelial cells, especially HUVE cells, have been described as 1,000 times less sensitive to endotoxin than bovine endothelial cells (37). However, we found that HUVE cells responded to 16K PRL (10 nM) and TNFα (5 ng/ml) in a similar manner, and to a lesser degree to endotoxin-1 (10 EU/ml). We also demonstrated that the response to 16K hPRL was specific for endothelial cells, while endotoxin and TNFα activated DNA fragmentation in endothelial cells as well as stromal and epithelial cells. Most importantly, the ability of 16K hPRL to activate DNA fragmentation in both bovine and human endothelial cells was abolished by immunoneutralization using specific monoclonal antibodies. The unlikely possibility that the activity of the recombinant preparations is due to some unknown heat-sensitive contaminant that co-precipitates with 16K hPRL cannot be ruled out.

Within 15 min 16K hPRL treatment resulted in conversion of the inactive proforms of caspase 1 and 3 to their active fragments. Activation of caspase 3 was directly confirmed by the cleavage and inactivation of the substrates, PARP and ICAD. Cleavage of both PARP (38) and ICAD (31, 39) by caspase 3 have been shown to be important steps in the apoptotic pathway. Inactivation of ICAD allows caspase-activated DNase (CAD) to enter the nucleus and degrade genomic DNA, while inactivation of PARP inhibits its DNA repair activity. The combination of these two events facilitates cellular disassembly and ensures the completion and commitment of the cell to the apoptotic pathway. Activation of the caspase cascade was essential for the apoptotic action of 16K hPRL. The processing of caspase 1 and the stimulation of DNA fragmentation by 16K hPRL were totally blocked by treatment with the caspase inhibitor, z-VAD.

Although the levels of Bcl-2, Bax, and Bak were unaffected by treatment with 16K hPRL, the conversion of Bcl-X was stimulated from its antiapoptotic form, Bcl-X_L, to its proapoptotic form, Bcl-X_s. Increased expression of Bcl-X_L appears to play an important role in the ability of nuclear factor (NF)-κB to inhibit apoptosis (40). The inhibition of the antiapoptotic action of Bcl-X_L by 16K hPRL treatment is consistent with its stimulation of apoptosis.

The signaling mechanisms mediating the multiple antiangiogenic actions of 16K hPRL on vascular endothelial cells are still poorly understood. Although a specific, high-affinity, saturable binding site for 16K hPRL has been described on capillary endothelial cells (15), the identity of the receptor molecule is unknown. The effects of 16K hPRL are not mediated via an action on the PRL receptor as an agonist or antagonist. As with the effects of 16K hPRL on cell proliferation (11) and stimulation of PAI-1 expression (19), intact 23-kDa hPRL has no effect on stimulating apoptosis or inhibiting the ability of 16K hPRL to stimulate apoptosis. The question of how binding of 16K hPRL to its receptor activates the caspase cascade and apoptosis, inhibits activation of Ras and presumably cell proliferation, and increases the expression of PAI-1 remains unclear. However, activation of multiple signaling pathways appears to be the rule rather than the exception with ligands acting on endothelial cells. For example, another antiangiogenic factor throm-bospondin inhibits endothelial cell proliferation and stimulates apoptosis (21, 41, 42). Several other cytokines have also been shown to affect multiple signaling pathways in endothelial cells, e.g. TNFα and transforming growth factor-β (TGFβ) (43-45).

Tumor growth and progression have been shown to be dependent on development of a new microvascu-lature (2). Inhibition of the action of the angiogenic factor VEGF has been shown to result in tumor endothelial cell apoptosis and tumor necrosis (46). As previously seen, withdrawal of bFGF and/or serum induces endothelial cell apoptosis *in vitro* (47). These observations support the importance of the regulation of apoptosis as a control mechanism for tumor angio-genesis. The ability of 16K hPRL to inhibit the stimulation of endothelial cell proliferation by the angiogenic factors VEGF and bFGF and to directly stimulate apoptosis strongly supports the potential of 16K hPRL as an antitumor drug.

In conclusion, we have clearly demonstrated that 16K hPRL protein in our recombinant preparation is responsible for promoting programmed cell death of cultured endothelial cells. Furthermore, we have presented detailed functional evidence that the caspase cascade is fundamental for 16K hPRL-induced apoptosis in a dose- and time-dependent manner. These findings reveal a novel and important mechanism for 16K hPRL to regulate angiogenesis and emphasize its potential antitumor properties.

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MATERIALS AND METHODS

Production of Recombinant Proteins

For the production of recombinant 16K hPRL the cDNA encoding hPRL minus the corresponding signal peptide was inserted into the pT7L expression vector (48). An ATG was genetically engineered 5' to the first codon of the cDNA. The codon for Cys 58 (TGC) was mutated to a Ser codon (TCC) to prevent the formation of incorrect disulfide bounds during refolding of the 16K hPRL (11). To get high levels of expression of the recombinant protein in *E. coli* the full-length hPRL molecule was expressed, purified, and then cleaved (14). To accomplish this the nucleotide sequence coding for amino acids 139-144 (Pro-Glu-Thr-Lys-Glu-Asn: CCT-GAA-ACC-AAA-GAA-AAT) in hPRL was replaced with the nucleotide sequence coding for the cleavage site of IgA protease (Pro-Arg-Pro-Pro-Thr-Pro: CCT-AGA-CCC-CCA-ACA-CCT). Cleavage occurred between Pro 142 and Thr 143.

In brief, intact protein expression was induced in *E. coli* BL21 DE3 with isopropyl β-D-thiogalactopyranoside and the inclusion bodies were isolated. After washing, the inclusion bodies were solubilized in denaturation buffer (8 M urea, 20 mM ethanolamine-HCI, pH 10, 1% 2-mercaptoethanol, 0.5 mM PMSF) for 10 min at 55 C and overnight at room temperature. After renaturation by dialysis against 20 mM ethanolamine-HCI, pH 9, the proteins were purified as previously described (48). The mutated hPRL was enzymatically cleaved with IgA protease (0.05%, 25 C, overnight; Roche Molecular Chemicals, Indianapolis, IN). The 16K hPRL was purified by ion-exchange chromatography (HiTrap Q, Pharmacia Biotech, Piscataway, NJ).

The purity of each recombinant protein exceeded 95% as estimated by silver staining. The *Limulus* amoebocyte lysate assay (E-Toxate kit, Sigma) was used to detect and quantify endotoxin levels. The endotoxin level of the 16K hPRL preparation used in the studies was 47×10^{-6} EU/ng protein.

Cell Culture

BBE and BAE cells were isolated as previously described (Refs. 49 and 50), respectively. The cells were grown and serially passaged in low glucose DMEM supplemented with 10% calf serum (CS), 2 ITIM L-glutamine, and antibiotics (100 U of penicillin/streptomycin per ml and 2.5 mg of fungizone per ml). Recombinant human basic FGF (bFGF, Promega Corp., Madison, Wl) was added (1 ng/ml) to the cultures every other day. Experiments were initiated with confluent cells between passages 6-12. Primary human umbilical vein endothelial (HUVE) cells were obtained from Clonetics Corp. (San Diego, CA), grown according to specification, and retained for up to six passages. Primary human endometrial stromal cells and human endometrial epithelial cell line (Ish-ikawa) were kindly provided by Drs. R. N. Taylor and D. Lebovic (University of California, San Francisco). Primary stromal cells were prepared and cultured from endometrial biopsies as described (51). Ishikawa cells were routinely grown in DMEM/F-12 (1:1) supplemented with 10% FBS, penicillin-streptomycin, and sodium pyruvate.

Cell Stimulation and Preparation of Cell Extracts

Confluent cell cultures were dispersed and plated at the density of $15\text{-}20 \times 10^3$ cells/cm^2 culture plate (one plate per condition) in appropriate media. Thirty six hours after plating, cells were either serum starved or left in medium containing 10% serum for another 24 h. Cells were left untreated or treated with 2 nM recombinant human $VEGF_{165}$ (VEGF, Genentech, Inc., South San Francisco, CA), 2 nM VEGF plus 2 nM 16K hPRL, 0.5 nM bFGF (Promega Corp.), 0.5 nM bFGF plus 2 nM 16K hPRL, various concentrations of 16K hPRL, recombinant human TNFα (Pepro Tech, Rocky Hill, NJ), en-dotoxin-1 (*Limulus amoebocyte* lysate, E-Toxate kit, Sigma) or endotoxin-2 (*Salmonella minnesota* Re 595, a minimal naturally occurring endotoxic structure of LPS, Sigma) for 24 h.

Incubations were terminated by aspiration of the medium, two washes with ice-cold PBS, and addition of 200 μl of lysis buffer (1% Triton X-100 lysis buffer containing 20 ITIM Tris-HCI, pH 8.0, 137 ITIM NaCl, 10% (vol/vol) glycerol, 2 mM EDTA, with (for detection of nuclear proteins) or without 3 M urea, 1 mM pefabloc, 0.14 U aprotinin, 20 μM leupeptin, and 1 mM sodium orthovanadate) at 4 C as previously described (16).

The peptidyl fluoromethyl ketone (fmk) caspase inhibitor z-VAD-fmk (20 μM, Enzyme Systems Products, Livermore, CA) was added at the time cells were serum deprived and/or treatment added.

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Endotoxin Controls

To study the effect of the addition of endotoxin (LPS), several approaches were used to demonstrate that the apoptotic activity of 16K hPRL preparations was not due to endotoxin contamination. Polymyxin-B (Sigma), an antibiotic that binds and inactivates LPS, was preincubated with samples for 10 min at 37 C in a final concentration of 10 μg/ml. Alternatively, 16K hPRL activity was destroyed in the sample to be added to the culture cells by boiling for 2 min, or proteolytic digestion with trypsin (Sigma) for 16 h at 37 C according to the manufacturer's protocol. Efficiency of the digestion was tested by Coomassie blue staining (Bio-Rad Laboratories, Inc.) of SDS-PAGE gels (16% acrylamide). Finally 16K hPRL activity was immunoneutralized by preincubation for 45 min at 4 C in serum free medium containing either a specific monoclonal antibody for 16K hPRL (4G7, Dr. Martial), a monoclonal antibody that recognizes both the 23 kDa native hPRL and 16K hPRL with equal potency (6E4, Dr. Martial) and, as controls, an unrelated monoclonal antibody (pp54) or a neutralizing monoclonal antibody antihuman TNFα (Upstate Biotechnology, Inc., Lake Placid, NY; 1:750 dilution).

DNA Fragmentation ELISA Assay

A hallmark of programmed cell death by apoptosis is the formation of multinucleosomal sized genomic DNA fragments. DNA fragments are multiples of 180 bp subunits associated with core histones. Accumulation of mono- and oligonucleosomes into the cytoplasm of apoptotic cells is due to the fact that DNA fragmentation occurs before the breakdown of the plasma membrane, which is not the case with cell death by necrosis. The levels of mono- and oligo-nucleosomal DNA released in the cytosol of apoptotic cells were measured using the Cell Death Detection ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN). This is a quantitative sandwich-enzyme-immunoassay using antibodies against DNA and histones. Levels of DNA fragmentation were expressed as an enrichment factor, calculated by dividing the absorbance of a given sample by the absorbance of the corresponding 10% CS control.

DNA Content Analysis

BBE cells were cultured and treated as described above. The cells were harvested by trypsinization, washed in cold PBS, and fixed in 80% ethanol in PBS at 4 C for 30 min. After centrifugation, the cell pellets were suspended into PBS and passed through a 70 μm nylon cell strainer (Falcon). The cells were stained with 10 μg/ml propidium iodide and treated with 200 μg/ml RNase A at 37 C for 30 min. The fluorescence of individual cells was measured with a FACScalibur cytoflu-orometer equipped with the CellQuest software (Becton Dickinson and Co., Franklin Lakes, NJ).

Western Blotting

To detect processing of apoptotic proteases (caspases 1 and 3), the cleavage of poly(ADP-ribose) polymerase (PARP), and to survey the protein expression of ICAD, Bax, Bak, Bcl2, Bcl-X, equal quantities of lysates from BBE cells were resolved by SDS/PAGE (8%, 12%, or 15%) and transferred to polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA). Separate Western blots were performed using a variety of antibodies including an antihuman caspase 1 (ICE) rabbit polyclonal antibody (Upstate Biotechnology, Inc. 1:1000 dilution) that recognizes the proenzyme of 45 kDa, the partially cleaved inactive form of 30 kDa, and the p20 subunit of active caspase 1 ; an antihuman caspase 3 (YAMA/Apopain/CPP32) rabbit polyclonal antibody (Upstate Biotechnology, Inc. 1:1000 dilution; Santa Cruz Biotechnology, Inc., 1:500 dilution) that recognizes the caspase 3 precursor (32 kDa), the partially processed inactive form of 28 kDa, and the p17 subunit of the active caspase 3; an antihuman PARP rabbit polyclonal antibody (Upstate Biotechnology, Inc. 1:750 dilution) that recognizes both the 116 kDa form of active PARP and its 85 kDa proteolytic fragment; an antihuman DFF-45/ ICAD rabbit polyclonal antibody (Upstate Biotechnology, Inc. 1:1000 dilution) that recognizes the N-terminal part of both the full-length molecule (45 kDa) and the caspase 3 generated form of approximately 34 kDa; an antihuman Bcl-2 oncoprotein mouse monoclonal antibody (Upstate Biotechnology, Inc. 1:500; Santa Cruz Biotechnology, Inc., 1:500 dilution) that recognizes a protein of 26 kDa; an antihuman Bak rabbit polyclonal antibody (Upstate Biotechnology, Inc. 1:1000 dilution) that recognizes a protein of 29 kDa; an antihuman Bax rabbit polyclonal antibody (Upstate Biotechnology, Inc. 1:500 dilution) that recognizes a protein of 23 kDa; and an antichicken Bcl-X_L/X_s rabbit polyclonal antibody (Upstate Biotechnology, Inc. 1:1000 dilution) that recognizes both proteins of 21 (Bcl-X_s) and 29 kDa (Bcl-X_L). Nuclear extract of human Hela, human A431 cells stimulated with or without EGF, and mouse 3T3 cell lysates were used appropriately as positive controls for all antibodies. Western blots were incubated with the appropriate antibody and then washed in Tris-buffered saline containing 0.1% Tween 20. Antigen-antibody complexes were detected with horseradish peroxidase-coupled

secondary antibodies and the enhanced chemiluminescence system (Renaissance, NEN Life Science Products, Boston, MA). Finally, the blots were developed on reflection NEF film (NEN Life Science Products).

Statistical Analysis

All values are expressed as mean ± SD. Comparisons between treatment conditions were assessed by one-way ANOVA with the post hoc analysis with the Student-Newman-Keuls test. Statistical significance was defined as a value of $P < 0.05$.

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