# Apoptosis of Mortal Human Fibroblasts Transformed by the Bovine Papillomavirus E5 Oncoprotein

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## Abstract

**Mortal human fibroblasts can be partially transformed by the bovine papillomavirus E5 oncoprotein through activation of the platelet-derived growth factor β receptor. Here, we report that these cells undergo massive apoptosis 2 weeks after confluence. Although activation of caspase 3 was observed in the apoptotic cells, it was not required for apoptosis. The appearance of the mitochondrial proteins cytochrome *c* and apoptosis-inducing factor in cytosolic and nuclear compartments, respectively, provided a basis for mitochondrial dysfunction and a caspase-independent mechanism of apoptosis in these cells. Although an activating conformational change in Bax also was evident in the apoptotic cells, enforced overexpression of Bcl-2 was insufficient to prevent apoptosis. Finally, a small peptide present in the conditioned medium from dying transformed cells appeared responsible for inducing apoptosis through affecting a conformational change in Bax and eventual relocalization of apoptosis-inducing factor to the nucleus. Thus, an atypical apoptotic pathway is activated in mortal human fibroblasts in response to transformation induced by sustained receptor tyrosine kinase activation.**

## Introduction

The E5 oncogene of bovine papillomavirus (BPV) encodes a small transmembrane protein that is capable of transforming rodent fibroblast lines to tumorigenicity (1–5). The E5 protein is a disulfide-linked homodimer with a subunit size of 7 kDa and localizes primarily to intracytoplasmic membranes in BPV-transformed cells (6). Through specific transmembrane interactions, the E5 protein forms a stable complex with the cellular β receptor for platelet-derived growth factor (PDGFβR) and induces its activation (7–9). Constitutive activation of this receptor tyrosine kinase is a key event mediating the transforming effects of E5 (10, 11). Evidence suggests that the E5 protein is able to activate the PDGFβR because of its dimeric nature; binding to the PDGFβR as a dimer allows the E5 protein to promote receptor dimerization, which in turn leads to receptor activation (12).

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We previously described the effects of expressing E5 in mortal human diploid fibroblasts (HDFs), which lack the accumulated genetic changes that are present in immortal rodent cells (13). We showed that the E5-expressing HDFs, like the previously described rodent fibroblast lines expressing E5, were morphologically transformed, capable of focus formation, and reached a higher saturation density than control cells. As expected, activation of the PDGFβR was observed in these cells and was required for the transforming effects of E5. However, unlike the rodent fibroblasts transformed by E5, the E5-expressing HDFs were neither immortalized, anchorage independent, or able to grow significantly in low serum, indicating that they lacked some of the characteristics of the fully transformed phenotype. Moreover, we found that when the E5-expressing HDFs reached confluence, growth inhibition accompanied by rapid degradation of the PDGFβR and retinoblastoma protein (pRb) was induced by a factor secreted in the medium. We proposed that an intracellular proteolysis pathway resulting in growth inhibition was activated at confluence as a negative feedback mechanism to limit the proliferative effects of sustained PDGFβR activation in these cells.

Apoptosis is defined as programmed cell death and is characterized by a cascade of genetic and biochemical events leading to cell shrinkage, condensation of cytoplasmic and nuclear materials, cytoplasmic membrane blebbing, formation of small apoptotic bodies, and cleavage of chromosomal DNA. Preliminary results from studies with the E5-transformed HDFs suggested that these cells might be undergoing a limited amount of apoptosis at confluence (13). First, after these cells reached their peak density, a slight decrease in the number of viable cells was typically observed. Also, subconfluent cells treated with conditioned medium from confluent E5-transformed HDFs displayed a slight decrease in viability. Finally, a 68-kDa form of Rb, previously shown to be present in apoptotic cells (14, 15), was evident in the E5-transformed HDFs after they reached confluence. Here, we demonstrate that the E5-transformed HDFs maintained without a medium change undergo massive apoptosis 2 weeks after confluence. Untransformed HDFs can survive for almost 2 months under the same conditions. We provide evidence to suggest that the transformed cells produce a small toxic peptide, which induces caspase-independent apoptosis by affecting mitochondrial dysfunction in a Bcl-2-resistant manner. Thus, apoptosis may serve as another control mechanism utilized by mortal human cells to hinder the oncogenic effects of sustained receptor tyrosine kinase signaling.

## Results

### *Apoptosis of the E5-Transformed HDFs*

HSF4012 cells (NHDF4012; Clonetics, San Diego, CA), a mortal human fibroblast cell strain with a normal diploid karyotype, were made to stably express the BPV E5 protein by

retroviral mediated gene transfer as previously described (13). Untransformed control cells expressing the retroviral vector without an insert were established in parallel. As we previously showed, the E5-expressing HDFs display some but not all of the characteristics of the fully transformed phenotype (13). The transforming phenotypes of these cells include morphologic transformation, focus formation, and an increased proliferative capacity, all of which require the kinase activity of the PDGFβR. However, these cells are not immortalized, anchorage independent, or able to grow significantly in low serum. In addition, growth inhibition accompanied by rapid degradation of the PDGFβR and pRb occurs when the E5-expressing HDFs reach confluence, and all three events are induced by a factor in the conditioned medium.

Here, we examined the effect of maintaining these cells at confluence without a medium change to determine the consequence of prolonged exposure to this putative growth-inhibiting factor. We observed that the E5-transformed HDFs died as an entire culture 2 weeks after confluence was reached (Figs. 1, 2, and 8). In contrast, untransformed control cells could survive as long as 50–60 days under the same conditions (*i.e.*, without medium replacement). The dead E5-transfomed HDFs appeared shrunken, remained adherent to the culture dish, and consisted of elongated cytoplasmic extensions as well as many highly refractile rounded structures that had the appearance of apoptotic bodies (Figs. 2 and 8B). A trypan blue dye exclusion assay on the adherent E5-transformed cells confirmed that the viability of these cells rapidly declined between 13 and 15 days postconfluence (Fig. 1A). Furthermore, the timing of the onset of cell death was the same for several different independently derived E5-transformed cell strains examined, consistently occurring between 13 and 14 days after confluence. A similar cell death phenomenon was also observed for HDFs transformed by *v-sis*, which encodes a viral homologue of the PDGFB isoform (data not shown, Fig. 1B). Thus, it appears that a cell death pathway is activated in transformed HDFs 2 weeks after confluence, resulting in death of the entire culture within 48 h.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) analysis, which allows the *in situ* detection of apoptotic DNA fragmentation, was performed to determine if a significant proportion of the E5- and *v-sis*-transformed HDFs had undergone apoptosis 2 weeks after confluence. The E5- and *v-sis*-transformed HDFs as well as the untransformed control cells were fixed 7 and 14 days after confluence and then subjected to TUNEL followed by 4′,6-diamidino-2-phenylindole (DAPI) staining. Both TUNEL- and DAPI-stained cells were counted and the fraction of total cells that were TUNEL-positive is illustrated in Fig. 1B. One week after confluence, only a small fraction of the control, E5- and *v-sis*-transformed cells exhibited TUNEL staining, consistent with the observation that all three cell types appeared viable at this time. One week later, although the number of TUNEL-positive control cells increased slightly, 70% of both the E5- and *v-sis*-transformed HDFs displayed bright TUNEL staining. Thus, a large fraction of the E5- and *v-sis*-transformed cells died via apoptosis.

We also monitored the morphological changes of the dying E5-transformed HDFs in more detail by time-lapse video microscopy. Videotaping of these cells was initiated when they first displayed the morphological signs of death and continued for 48 h thereafter. Fig. 2 shows six time frames of the same field captured at 1-h intervals. As indicated by the *arrows*, one can track the formation of small protrusions at the end of cytoplasmic extensions that eventually detach from the cell and give rise to small rounded structures. Subsequent cleavage of these rounded structures is also evident. This entire process resembles a phenomenon known as "membrane blebbing," which is thought to result from cell surface convolution and is typical of apoptotic cells. Thus, the dying transformed HDFs appeared to undergo the morphological changes that are characteristic of apoptotic cells.

### *The Effect of Certain Kinase Inhibitors on Apoptosis of the Transformed HDFs*

We previously reported that the E5 protein transforms mortal human fibroblasts through its ability to bind to and constitutively activate the PDGFβR, because AG1296, a kinase inhibitor specific for this receptor (16), inhibited transformation

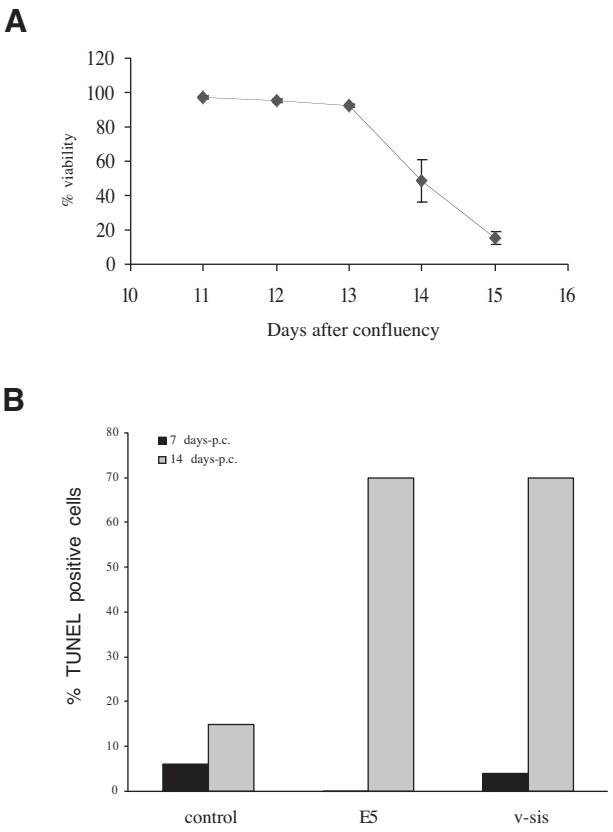


**FIGURE 1.** Viability and TUNEL analysis of the E5-transformed HDFs. **A.** The viability of E5-transformed HDFs between 11 and 15 days after confluence was determined by a trypan blue viability assay. For each time point, cells were counted in triplicate, and the mean value for the percentage of live cells with the SD was plotted. **B.** Control, E5-, or *v-sis*-transformed HDFs were fixed 7 or 14 days postconfluence (*p.c.*) and then subjected to TUNEL followed by DAPI staining. Stained cells were examined by fluorescent microscopy, and images were captured using a digital camera interfaced with a fluorescence microscope and analyzed using the Scion Image (Scion Corp., Fredrick, MD) program. For each sample, both TUNEL- and DAPI-stained cells were counted in multiple frames, and the percentage TUNEL-positive cells was calculated from the average number of TUNEL-stained cells per DAPI-stained cells. Between 560 and 1700 cells were counted for each sample.

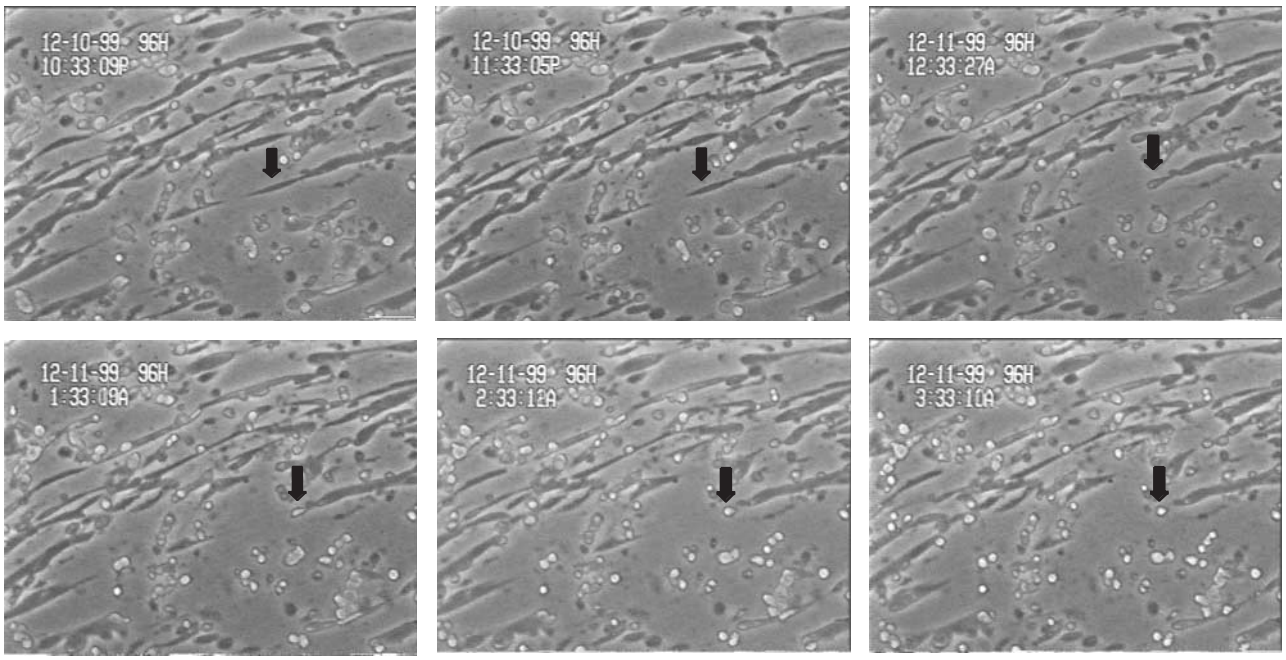


**FIGURE 2.** Time-lapse video microscopy of dying E5-transformed HDFs. Each panel depicts the same field of cells videotaped for 5 h and captured at 1-h intervals. *Black arrows* indicate apparent membrane blebbing typical for apoptotic cells.

of HDFs by E5 (13). However, inhibition of transformation was only effective if the cells were treated with AG1296 before confluence. The rapid degradation of the PDGFβR that was observed at confluence also could be inhibited by AG1296, but only when the inhibitor was added before the onset of this degradation [(13); Fig. 3]. Here, we found that if AG1296 was added to the cells before they reached confluence, it inhibited cell death and allowed the cells to live as long as the control cells, *i.e.*, 50–60 days (Fig. 3B, Table 1). AG1296 treatment any time after confluence had no inhibitory effect on apoptosis. Thus, constitutive activation of the PDGFβR in the E5-transformed HDFs before confluence was required for apoptosis 2 weeks later. Like AG1296, LY294002, an inhibitor specific for phosphatidylinositol (PI) 3-kinase (17), completely prevented PDGFβR degradation at confluence as well as apoptosis when added to the cells before but not after confluence (Fig. 3, Table 1). This suggests that PI 3-kinase activity before confluence also was required for apoptosis of these cells. Cells treated with AG1296 or LY294002 just before confluence still reached their optimal saturation density, and were protected from apoptosis. A similar situation was observed for cells maintained in serum-free medium after confluence; an optimal saturation density was achieved but apoptosis was completely prevented (see below). Thus, apoptosis can be separated from and hence does not result from maintenance of cells at high density. PD98059, an inhibitor of the mitogen-activated protein (MAP) kinase pathway (18), when added to the cells any time before or after confluence, could not prevent apoptosis, nor could it completely inhibit PDGFβR degradation at confluence (Table 1, Fig. 3A). Another MAP kinase pathway inhibitor, apigenin (19) and a p38 kinase inhibitor, SB203580 (20), also were unable to protect against apoptosis (Table 1). Taken together, these results suggest that activation of only particular PDGFβR signaling pathways, such as the PI 3-kinase pathway, before confluence may be required for apoptosis of the E5-transformed HDFs. Furthermore, these results also provide a correlation between PDGFβR degradation at confluence and apoptosis 2 weeks later.

### *Caspase 3 Activation and Its Role in Apoptosis of the E5-Transformed Cells*

We also examined the E5-transformed HDFs for activation of caspases, cytoplasmic cysteine proteases that cleave after an aspartatic acid residue and are mediators of a major apoptotic pathway [reviewed in Ref. (21)]. Caspase 3, a major effector caspase, is synthesized as a 33-kDa inactive proenzyme and during apoptosis is activated by autoproteolysis or cleavage by other caspases to generate a 17-kDa active enzyme. One important substrate of caspase 3 is poly(ADP-ribose) polymerase (PARP), which plays an important role in repair of double-stranded DNA breaks as part of the normal cellular response to DNA damage [reviewed in Ref. (21)]. During apoptosis, PARP is cleaved by caspase 3 from its 116-kDa intact active form into two inactive fragments of 85 and 25 kDa. Therefore, such caspase 3 and PARP cleavage events are indicative of caspase 3 activation.

To detect the presence of caspase 3 or PARP cleavage products in the transformed fibroblasts, whole cell lysates of live, dying, and dead cells were subjected to immunoblot analysis for caspase 3 and PARP. As shown in Fig. 4, a 17-kDa cleavage product of caspase 3 could be detected (along with reduced levels of the 33-kDa proenzyme) in the dying and dead cells (*lanes 2–4*), but not in the live cells (*lanes 1*, *5*, and *6*). This indicated that caspase 3 was activated when these cells began to die. Similarly, an 85-kDa form of PARP was apparent along with

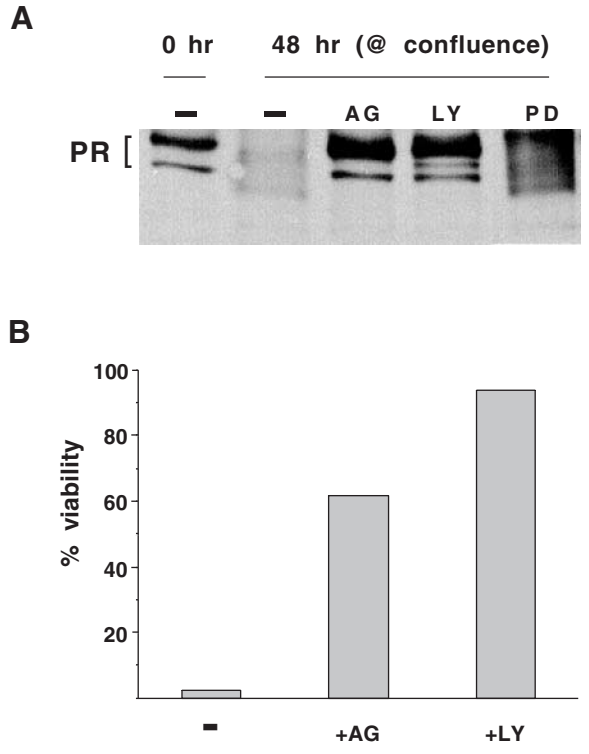


**FIGURE 3.** The effect of kinase inhibitors on PDGFβR degradation at confluence and apoptosis in the E5-transformed HDFs. Before confluence, E5-transformed HDFs in 60-mm dishes were either untreated (–) or treated with the kinase inhibitors AG1296 (*AG*), LY294002 (*LY*), or PD98059 (*PD*) by addition of the inhibitor directly to the medium at final concentrations of 17.5, 20, or 50 μM, respectively. **A.** Two days later, when the cells reached confluence, cells were lysed, and whole cell extracts were subjected to PDGFβR immunoblotting. Approximately equal amounts of protein were loaded in each lane as determined by Ponceau S (Sigma, St. Louis, MO) staining before immunoblotting (data not shown). **B.** Two weeks after inhibitor treatment, when the untreated cells appeared apoptotic, cell viability was determined by a trypan blue dye exclusion assay.

reduced levels of the 116-kDa full-sized form in the dying and dead HDFs, but not in the live cells. This indicated that PARP also was cleaved in the dying cells, providing additional evidence for caspase 3 activation in the transformed HDFs.

To determine the role of caspase 3 in apoptosis of the E5-transformed HDFs, caspase inhibitors were used. DEVD-CHO, a caspase 3-specific inhibitor, or z-VAD-fmk, a general caspase inhibitor, was added to the medium of these cells at a final concentration of 100 μM a few days before confluence or before death. Surprisingly, neither inhibitor prevented death of the E5-transformed HDFs even though each inhibited caspase 3 cleavage in these cells (Table 1). These reagents were funcional apoptotic inhibitors because they were able to hinder apoptosis of IL-3-deprived Ba/F3 cells (data not shown), which normally depend on IL-3 for survival (22). Thus, despite evidence that caspase 3 was activated in the apoptotic E5-transformed HDFs, it does not appear to be essential for apoptosis of these cells.

### *Relocalization of Cytochrome c and Apoptosis-Inducing Factor (AIF) in the E5-Transformed HDFs*

Permeabilization of the inner mitochondrial membrane is thought to lead to apoptosis because it results in the release of apoptotic proteins such as cytochrome *c* and AIF into the cytosol. When released into the cytosol, cytochrome *c* binds to apoptosis protease-activating factor 1 (Apaf-1) and allows it to activate a caspase cascade culminating in internucleosomal (small-scale) DNA fragmentation (23). Release of AIF from the mitochondria into the cytosol is followed by its translocation to the nucleus where it is thought to induce large-scale DNA fragmentation (24–27). Unlike cytochrome *c*, apoptosis induced by AIF is caspase independent (25–27). We hypothesized, therefore, that both caspase-dependent and caspase-independent apoptotic pathways exist in the E5-transformed HDFs due to the activities of extramitochondrial cytochrome *c* and AIF, respectively.

To test this possibility, we first examined cytochrome *c* and AIF levels in cytosolic fractions of the transformed fibroblasts. Both whole cell and cytosolic extracts were prepared at various times before death and then subjected to cytochrome *c* or AIF immunoblotting (Fig. 5). Fig. 5A (*left*) shows that very little cytochrome *c* was present in the cytosol of untransformed postconfluent HDFs and the E5-transformed HDFs 12 days before death. However, 1 day later, the amount of cytosolic cytochrome *c* dramatically increased in the transformed HDFs and further increased with time as the cells approached death. The integral mitochondrial protein cytochrome *c* oxidase subunit II (COXII) could not be detected in these cytosolic fractions, indicating that they were relatively free of mitochondrial contamination. Thus, cytochrome *c* appeared to be released from the mitochondria into the cytosol of the transformed HDFs as early as 11 days before death. Total cellular levels of cytochrome *c* also gradually increased after confluence as the cells approached death (Fig. 5A, *right*).

Increased levels of a 57-kDa form of AIF as well as lower molecular weight forms appeared in cytosolic fractions of the E5-transformed HDFs 2–3 days before death (Fig. 5B, *left*). The 57-kDa AIF form most likely represents the mature form of AIF, which is generated by $NH_2$-terminal cleavage of the mitochondrial localization signal after translocation of a 67-kDa precursor form to the mitochondria (26). Since the 57-kDa mature AIF form should be associated primarily with the mitochondria in healthy cells, the presence of this form in cytosolic fractions may signify mature AIF that had been released from the mitochondria into the cytosol before death. The significance of the lower

**Table 1. The Effect of Various Inhibitors on Apoptosis of the E5-Transformed HDFs**

| Inhibitor | Target | μM[a] | Inhibition of Apoptosis |
|---|---|---|---|
| DEVD-CHO | Caspase 3 | 100 | no |
| z-VAD-fmk | Caspases | 100 | no |
| AG1296 | PDGF receptor | 17.5 | yes[b] |
| LY294002 | PI 3-kinase | 20 | yes[b] |
| PD98059 | MEK | 50 | no |
| Apigenin | MAP kinase | 36 | no |
| SB203580 | p38 | 1 | no |

[a]Each inhibitor was added directly to the cell culture medium at this final concentration.
[b]Inhibition of apoptosis was observed only when the inhibitor was added before confluence.

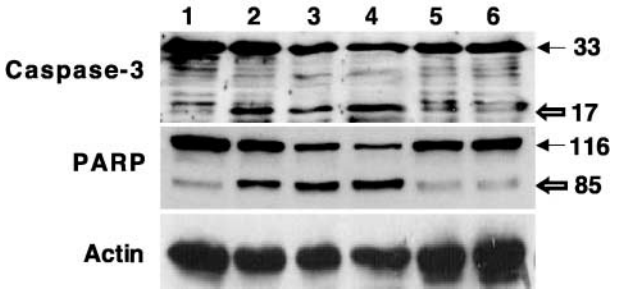


**FIGURE 4.** Caspase activation in the apoptotic HDFs. E5-transformed HDFs were maintained at confluence without a medium change for 12–14 days. Whole cell extracts of live cells 1 day before they began to die (*lane 1*), dying cells (*lane 2*), dead cells (*lanes 3* and *4*), or dying cells rescued by a medium change (*lanes 5* and *6*) were subjected to caspase 3, PARP, and actin immunoblotting. *Arrows* to the right indicate the size in kilodaltons of the unprocessed and cleaved forms of caspase 3 and PARP.

molecular weight forms of AIF in the cytosol is uncertain. Analysis of whole cell extracts revealed that the total amount of both the 67- and 57-kDa AIF forms gradually increased as the cells approached death (Fig. 5B, *right*).

Immunofluorescence analysis also allowed us to monitor the cellular distribution of cytochrome *c* and AIF before death (Fig. 6). Subconfluent cells displayed a thread-like punctate cytochrome *c* staining in the cytoplasm, indicating mitochondrial localization of cytochrome *c* at this time. Postconfluent cells exhibited large masses of intense cytoplasmic cytochrome *c* staining, suggesting that it had redistributed in the cytoplasm. This was even more apparent in the dying cells where many of these stained masses appeared to be perinuclear. AIF staining of subconfluent cells also appeared to be mitochondrial, as indicated by a punctate cytoplasmic staining pattern that excluded the nucleus. However, in the postconfluent cells, AIF staining was more diffuse and appeared to include the entire cell. In the dying cells, AIF staining was more intensely nuclear, as indicated by counterstaining the same cells with DAPI (*bottom panels* in Fig. 6). This indicates that AIF had translocated to the nucleus before death.

In sum, our results suggest that both AIF and cytochrome *c* were released from the mitochondria into the cytosol of the E5-transformed HDFs before death, and the total amount of both proteins gradually increased between confluence and death. Cytochrome *c* appeared to be released from the mitochondria and redistributed to discrete cytosolic compartments, whereas AIF was released into the cytosol and translocated to the nucleus. These results not only provide evidence for increased mitochondrial membrane permeability in the transformed HDFs, but also support the notion that both caspase-dependent and -independent apoptotic pathways are at work in these cells.

### *Analysis of Bcl-2-Related Proteins in the E5-Transformed HDFs*

To determine the basis for mitochondrial dysfunction in the E5-transformed HDFs, we searched for changes in levels of several pro- and anti-apoptotic Bcl-2 family proteins, which are thought to regulate mitochondrial membrane permeability. Immunoblot analysis was performed on whole cell extracts of postconfluent cells prepared 1–12 days before death. As shown in Fig. 7A, levels of the anti-apoptotic Bcl-2 protein unexpectedly increased in the transformed cells between 11 and 8 days before death. Levels of another anti-apoptotic protein, Bcl-$X_L$, and the proapoptotic protein, Bax, were similar in untransformed and transformed HDFs and remained unchanged in the transformed cells with time in culture as the cells approached death. Expression of the proapoptotic Bcl-$X_S$ was increased in the transformed cells compared to the untransformed cells.

Although the levels of Bax appeared unchanged in the E5-transformed cells, the possibility remained that the activity of Bax increased during apoptosis. Evidence suggests that a conformational change in Bax involving exposure of its $NH_2$- and COOH-terminal domains is required for its insertion into the mitochondrial outer membrane and hence its apoptotic activity (28–30). A previous report showed that this activating conformational change could be detected using the conformation-specific antibody 6A7, which recognizes an $NH_2$-terminal epitope that is hidden in the inactive form but exposed in the active form (30). To assess the activity of Bax in the transformed HDFs, we used the 6A7 antibody in immunofluorescence analysis. As illustrated in Fig. 7B, the 6A7 antibody produced a faint punctate cytoplasmic staining of subconfluent E5-transformed cells, indicating that a low level of Bax was activated in the mitochondria before confluence. In contrast, bright staining with this antibody was evident in the dying cells, indicating that levels of the activated form of Bax had substantially increased in the apoptotic cells. Thus, an apparent increase in the activity of Bax correlates with apoptosis of these cells.

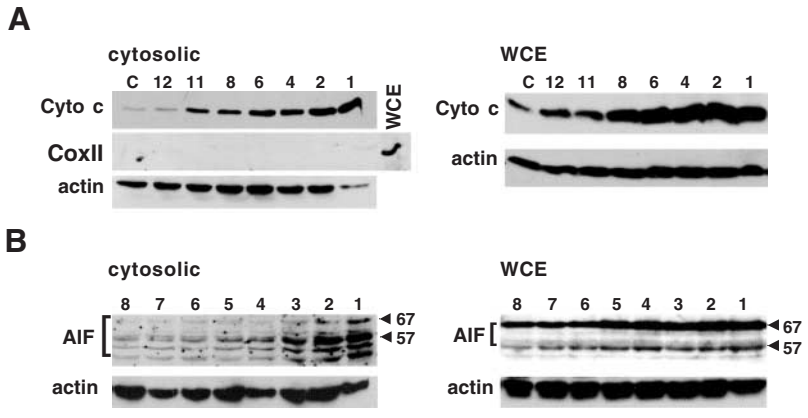


**FIGURE 5.** The presence of cytochrome *c* and AIF in cytosolic fractions of the E5-transformed HDFs. Cytosolic or whole cell extracts (*WCE*) of untransformed control or E5-transformed HDFs were prepared at various times before the cells began to die as described in "Materials and Methods." Immunoblot analysis for cytochrome *c*, (**A**), AIF (**B**), or the integral mitochondrial protein COXII (**A**) was performed. *Numbered lanes,* E5-transformed HDFs lysed at the indicated number of days before death; *lane 1,* cells that were beginning to show signs of apoptosis; *lane C,* extracts of confluent control cells. In panel **B**, *arrows* on the right point to AIF bands of 67 and 57 kDa, which are likely to correspond to the precursor and mature forms of AIF, respectively. Immunoblotting for actin levels also was performed as an indication of the amount of protein loaded in each lane.

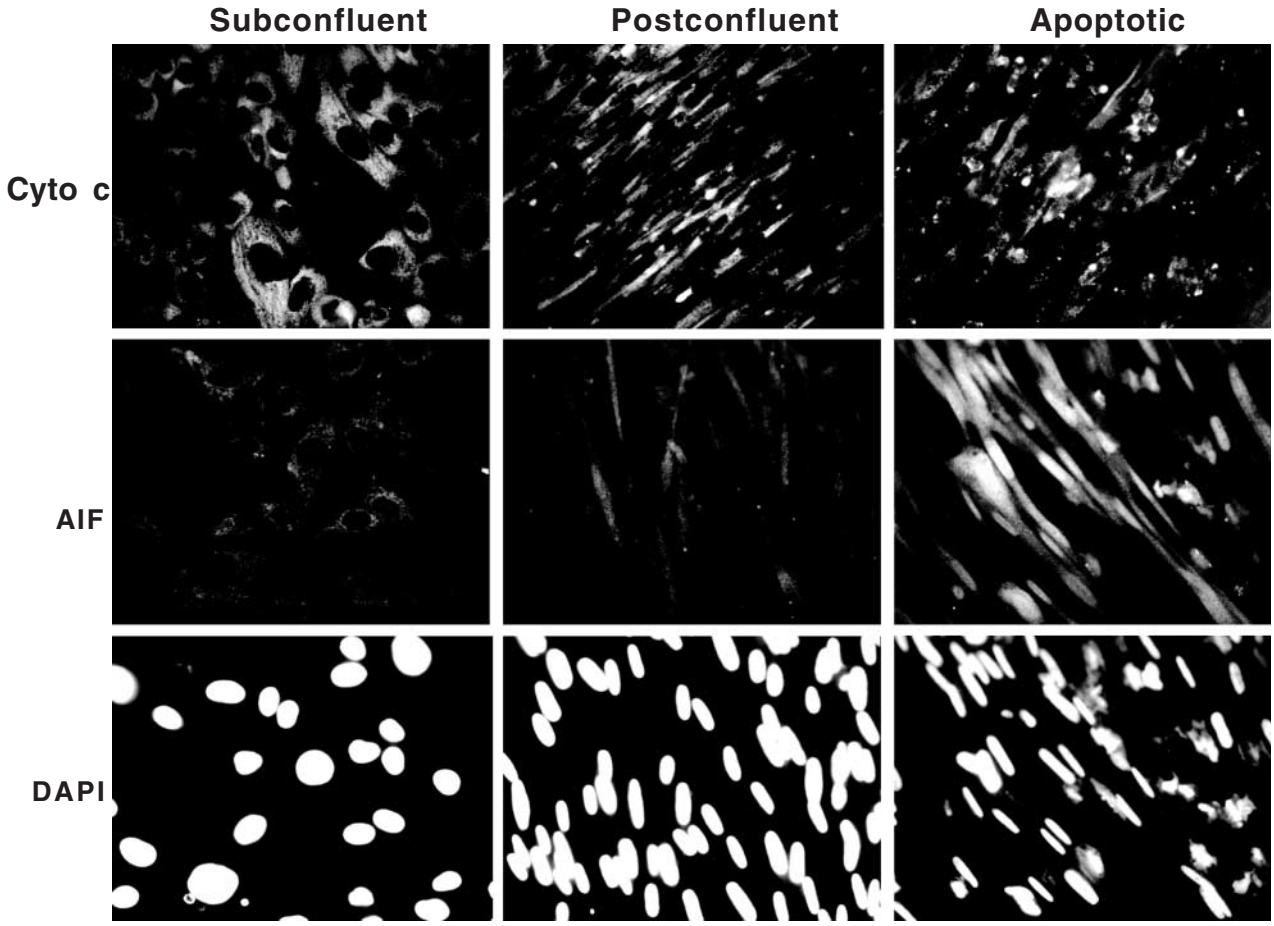


**FIGURE 6.** Immunofluorescence staining of cytochrome *c* and AIF in the E5-transformed HDFs. Subconfluent, postconfluent, or apoptotic E5-transformed HDFs that were grown on coverslips were fixed and then stained with cytochrome *c* (*Cyto c*) or AIF antibodies. Nuclei of the AIF-stained cells were visualized by counterstaining with DAPI. Images of cells were captured with a digital camera interfaced with a fluorescence microscope and analyzed using the Scion Image program (Scion Corp.).

*The Effect of Bcl-2 Overexpression on Apoptosis of the Transformed HDFs*

Apoptosis of the E5-transformed HDFs occurred despite an increase in Bcl-2 levels before death (Fig. 7A). To definitively determine whether or not Bcl-2 could effectively protect these cells against apoptosis, Bcl-2 was heterologously overexpressed in these cells. The human *bcl-2* cDNA was introduced into the control and E5-transformed HDFs by retroviral mediated gene transfer, and cells stably overexpressing Bcl-2 were established. Bcl-2 overexpression was confirmed by immunoblotting (Fig. 7C) and appeared to have no effect on the morphology or growth rate of these cells (data not shown). Moreover, Bcl-2 expressed in this manner was functional, because it protected against apoptosis induced by staurosporine treatment in untransformed HDFs and apoptosis induced by IL-3 deprivation in Ba/F3 cells. Surprisingly, transformed HDFs overexpressing Bcl-2 still died 2 weeks after confluence as did the transformed HDFs lacking exogenous Bcl-2. Other independently derived E5-transformed cell strains overexpressing Bcl-2 displayed the same kinetics of cell death. Therefore, overexpression of Bcl-2 could not protect these cells from undergoing apoptosis.

*Evidence for the Presence of a Toxic Factor in the Conditioned Medium of Apoptotic Transformed HDFs*

We next determined whether medium replacement or addition of growth factors could rescue the E5-transformed HDFs from apoptosis 2 weeks after confluence. When these cells were beginning to show signs of death after being maintained at confluence for nearly 2 weeks (Fig. 8A), their medium was replaced, or they were treated with specific growth factors. As expected, cells left untreated died 24 h later (Fig. 8B). However, medium replacement with fresh 10% fetal bovine serum (FBS)-containing medium (Fig. 8F) restored the morphology of the cells and rescued them from death. Caspase-3 and PARP cleavage also was reversed by medium replacement (Fig. 4, *lanes 5* and *6*). Addition of PDGF BB or FGF to the existing medium did not save the cells from death (Fig. 8, C and D), suggesting that apoptosis did not result from depletion of these particular growth factors. Moreover, medium replacement with fresh serum-free medium, which theoretically should lack survival factors, completely rescued the cells from apoptosis (Fig. 8E). Taken together, these results imply that medium replacement rescued the cells through removal of a toxic factor rather than by supplying essential survival factors

that had been depleted. Cells that were rescued by medium containing 10% FBS eventually died 2 weeks later unless the medium was similarly replaced again. In contrast, cells rescued by serum-free medium survived for as long as the control cells (*i.e.*, 50–60 days). Therefore, signals induced by serum in the medium are likely to play a role in apoptosis. Also, because high density, postconfluent cultures could be completely rescued from apoptosis by medium replacement with serum-free medium, this apoptosis is not a nonspecific effect of maintaining cells at a high density.

The possibility that the E5-transformed HDFs produce a toxic factor was further addressed by determining if the conditioned medium from dead E5-transformed HDFs could induce death of normal untransformed HSF4012 cells. HSF4012 cells cultured 2–3 days postconfluence had their medium replaced with conditioned medium from the dead E5-transformed cells. Strikingly, this conditioned medium induced death of the HSF4012 cells, typically within 1–3 days of treatment (Figs. 9 and 10B). Medium taken from normal untransformed HDFs 2 weeks after confluence displayed no death-inducing activity (data not shown). Overall, the HSF4012 cells treated with the apoptotic medium resembled the dead E5-transformed HDFs with fewer remnants of elongated cytoplasmic extensions. The apoptotic characteristic of membrane blebbing was particularly evident for these dead HSF4012 cells (Fig. 9B). Moreover, many of the dead HSF4012 cells displayed positive TUNEL staining (Fig. 9C) that was primarily nuclear, whereas the live untreated cells showed no TUNEL staining (data not shown). Thus, conditioned medium from the transformed HDFs had the ability to induce apoptosis of untransformed HDFs, providing further evidence that apoptosis of the transformed cells was induced in an autocrine manner by a toxic factor produced by the cells.

Apoptosis of the HSF4012 cells induced by the medium from the apoptotic E5-transformed HDFs appeared to be mechanistically similar to that of the E5-transformed cells. First, although the treated HSF4012 cells displayed evidence of caspase activation, they could not be rescued by pretreatment with a caspase 3-specific inhibitor (data not shown). In addition, enforced overexpression of Bcl-2 in HSF4012 cells afforded no protection against apoptosis induced by the conditioned medium (but did protect against apoptosis induced by staurosporine treatment). Thus, apoptosis induced by the putative toxic factor was caspase-3 independent as well as Bcl-2 resistant. Interestingly, pretreatment of HSF4012 cells with AG1296, the PDGFβR-specific kinase inhibitor, LY294002, the PI-3 kinase inhibitor, or PD98059, the MEK specific inhibitor, was unable to prevent death induced by the apoptotic medium (Fig. 9A). This suggested that PDGFβR, PI-3 kinase, and MEK activities were not required for apoptosis induced by the putative toxic factor. However, it is still possible that the PDGFβR and/or PI-3 kinase activities may be required for secretion of the toxic factor, because AG1296 and LY294002 were able to prevent apoptosis of the transformed HDFs when added before confluence as demonstrated above (Fig. 3B).

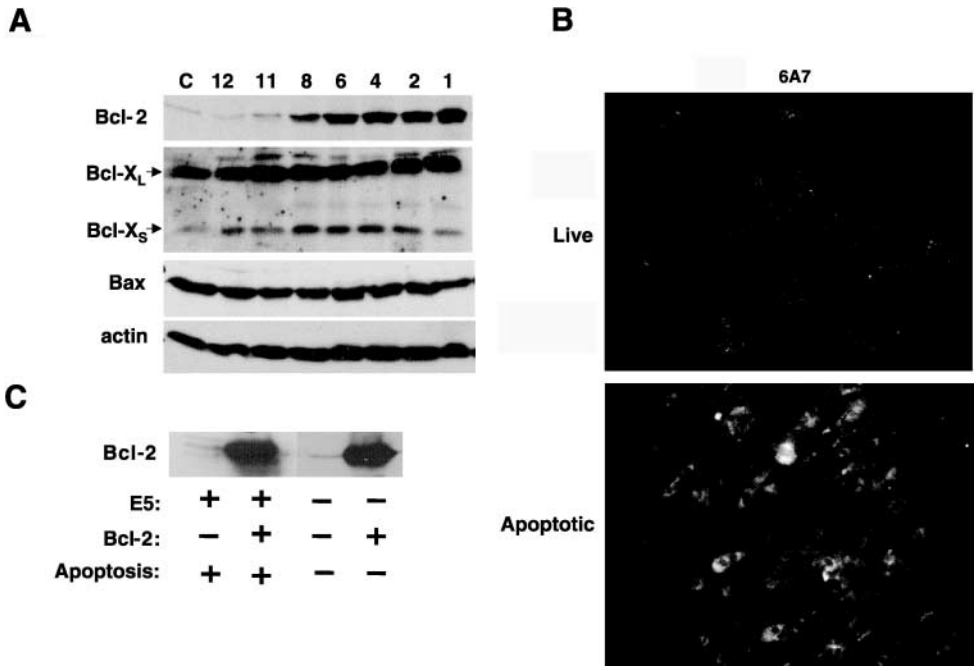


**FIGURE 7.** Analysis of Bcl-2-related proteins in the E5-transformed HDFs. **A.** Whole cell extracts of untransformed control or E5-transformed HDFs were prepared at various times before death and examined for Bcl-2, Bcl-$X_L$, Bcl-$X_S$, Bax, and actin levels by immunoblot analysis. *Top panels, lane C,* an extract of confluent control cells; *numbered lanes,* E5-transformed HDFs lysed at the indicated number of days before death. **B.** Immunofluorescence staining for the conformationally active form of Bax in the E5-transformed HDFs. Live subconfluent or apoptotic E5-transformed HDFs were fixed and stained with the conformation-specific anti-Bax antibody 6A7. Images of cells were captured using a digital camera interfaced with a fluorescence microscope and analyzed as in Fig. 6. **C.** Overexpression of Bcl-2 in HDFs. Untransformed control or E5-transformed HDFs stably overexpressing Bcl-2 or the corresponding empty vector were established as described in "Materials and Methods." Bcl-2 immunoblot analysis of whole cell extracts was performed to confirm Bcl-2 overexpression in the appropriate cell lines. The absence (−) or presence (+) of E5 or heterologous Bcl-2 expression in the cell lines tested is indicated. Apoptosis 2 weeks after confluence was evaluated and is indicated (+ or −).

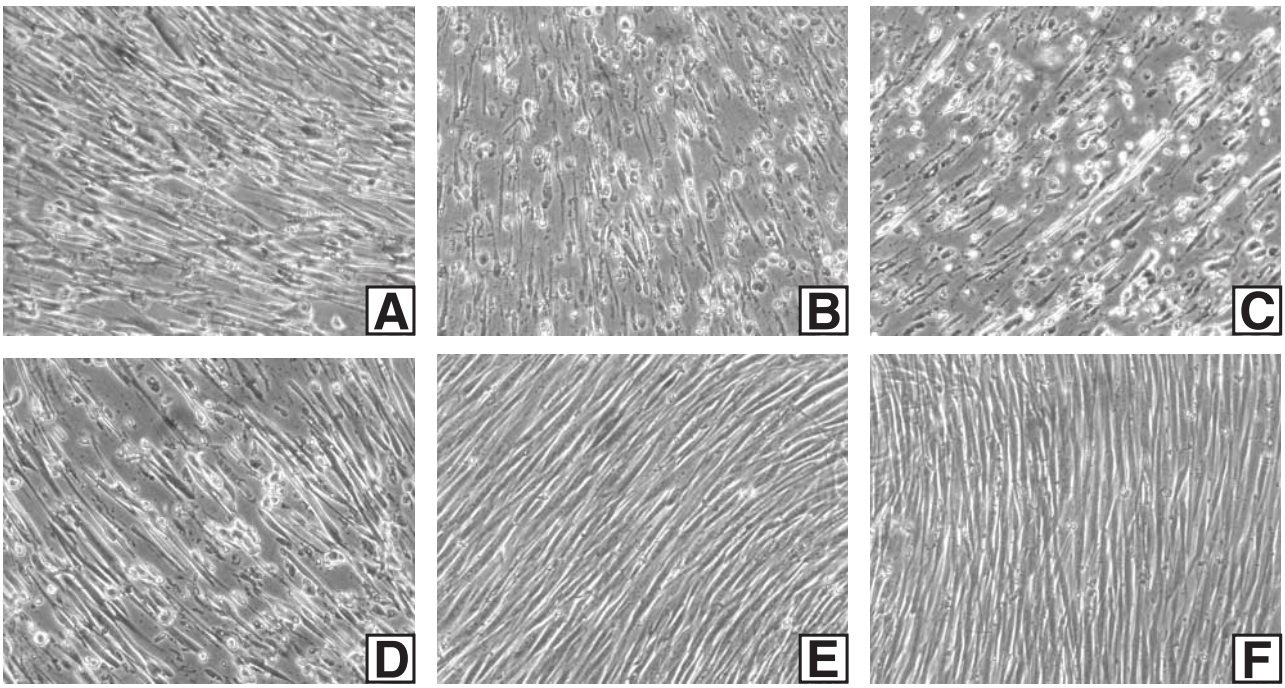


**FIGURE 8.** The effect of growth factors or medium replacement on dying E5-transformed HDFs. E5-transformed HDFs that were dying (**A**) were either left untreated (**B**), treated with 50 ng/ml PDGF BB (Collaborative Research, Bedford, MA) (**C**) or 2 ng/ml FGF (Clonetics) (**D**), or subjected to medium replacement with either serum-free (**E**) or 10% FBS-containing (**F**) medium. Immediately before (**A**) or 1 day after (**B**–**F**) the treatment, images were captured using a digital camera interfaced with an inverted microscope and analyzed using the Scion image program (Scion Corp.).

Conditioned medium from apoptotic E5-transformed HDFs also was able to induce death of a variety of tumor and non-tumor cell lines, including mouse C127 cells, human 293 cells, human MDA breast carcinoma cells, human MCF-7 breast carcinoma cells, and human SK-N-SH neuroblastoma cells. These cells died to the same extent and at a similar rate as normal HDFs that were treated in the same manner; *i.e.*, the entire culture typically died 1–3 days after medium replacement. Conditioned medium from normal untransformed HDFs maintained at confluence for 2 weeks did not have this apoptotic effect. Thus, the toxic factor secreted by the transformed HDFs does not appear to be species- or cell-type specific. Moreover, death induced by the toxic factor probably does not require the presence of the PDGFβR on the cell surface, because 293 cells do not express the PDGFβR. It is important to note that the MCF-7 breast carcinoma cell line has a functional deletion of the caspase 3 gene (31), but is still sensitive to conditioned medium from dead HDFs. This provides further evidence that the toxic factor secreted by the transformed HDFs stimulates a caspase 3-independent apoptotic pathway.

Medium removed from the E5-transformed HDFs 1–4 days before they died also could induce death of normal HSF4012 cells, although the death was delayed and prolonged. Specifically, medium taken from the transformed cells 1 day before death induced death of the normal HDFs within 4 days, representing a delay of only 1–2 days. However, medium taken from transformed HDFs 2, 3, and 4 days before they died caused a 3-, 6-, and 8-day delay, respectively, in the onset of death, and prolonged the length of time for the entire culture to die by 3–4 days. This progressive lag and slowing of cell death could be due to lower concentrations of a toxic factor in the conditioned medium. A similar effect was observed with increasing dilutions of the conditioned medium; dilution of the medium as little as 1:1 in PBS resulted in a 2- to 3-day delay of its apoptotic effect. Therefore, these results suggest that the toxic factor was gradually secreted from the transformed HDFs over a period of several days and accumulated until reaching a critical toxic concentration. It is also important to note that apoptosis required sustained treatment with the conditioned medium, because treatment for 2–16 h followed by replacement with fresh medium did not kill HSF4012 cells.

### *Initial Characterization of a Toxic Factor Produced by the E5-Transformed HDFs*

To initially characterize the putative toxic factor, conditioned medium from dead E5-transformed HDFs was subjected to various treatments and then tested for its ability to induce death of untransformed HDFs. First, medium that was boiled for 5 min before treatment had the same ability to induce death as untreated medium (compare Fig. 10, B and C), suggesting that the toxic factor is heat stable. Next, to roughly estimate the size of this factor, conditioned medium was fractionated using a centrifugal filter device with a molecular weight cut off pore size of 3 kDa. The filtrate fraction containing solutes smaller than 3 kDa still was able to efficiently induce death of normal HDFs (Fig. 10D), whereas fresh medium treated in the same way did not have this effect (data not shown). This suggests that the toxic factor was smaller than 3 kDa. In fact, preliminary results suggest that this factor may be 1 kDa or smaller because it appeared to

diffuse through a dialysis membrane with a pore size cut off of 1 kDa (data not shown). To determine whether this factor was a peptide, we assessed its sensitivity to protease treatment. Specifically, the apoptotic-conditioned medium was incubated with proteinase k at 37°C for 30 min. Because the proteinase k itself was toxic to the cells, the medium was boiled or fractionated through a centrifugal filter device after proteinase k treatment to either inactivate or remove, respectively, the proteinase k. As shown in Fig. 10, E and F, the proteinase k-treated medium was not able to kill the normal HDFs. Because boiling or fractionation did not affect the death-inducing activity in the medium (as described above, Fig. 10, C and D), these results suggest that the apoptotic factor was sensitive to the proteinase k treatment. This death-inducing activity in the medium was also sensitive to trypsin, chymotrypsin, and endoproteinase Glu-C (data not shown). Therefore, based on its sensitivity to protease digestion, low molecular weight, and heat stability, the toxic factor in the apoptotic-conditioned medium is likely to be a small peptide. Trypsin sensitivity indicates that the putative toxic peptide may contain lysine and/or arginine residues. Consistent with this, we found that the proteinase-sensitive apoptotic activity in the conditioned medium was excluded from a hydrophobic reverse-phase silica matrix, suggesting that the toxic peptide contains charged and/or polar residues.

### *Activation of Bax and Nuclear Translocation of AIF in Response to the Toxic Factor*

We next determined whether the conditioned medium from the apoptotic E5-transformed HDFs could induce a conformational change in Bax and nuclear translocation of AIF by immunofluorescence analysis. Briefly, normal HSF4012 cells were either untreated or had their medium replaced with conditioned medium from apoptotic E5-transformed cells. Four, 8, and 32 h later, the cells were fixed and stained for active Bax (using the conformation-specific 6A7 antibody) or AIF. As shown in Fig. 11, only a low level of fluorescence was observed in the untreated cells after staining with the 6A7 antibody, indicating that the majority of Bax expressed in these cells (Fig. 7, *lane C*) was in its conformationally inactive state. A substantial increase in the intensity of punctate cytoplasmic staining with the 6A7 antibody was evident as early as 4 h and even brighter by 8 h after treatment. Bright staining with this antibody was maintained after 32 h of treatment, at which time the cells appeared apoptotic. AIF staining of the untreated cells excluded the nuclei and was typical of a mitochondrial-staining pattern (as indicated by counterstaining with a mitochondrial dye; *lower left panels* of Fig. 11). A similar AIF staining pattern in which the nuclei were excluded also was observed after treatment with the apoptotic conditioned medium for 4 and 8 h. On the other hand, apoptotic cells that had been treated for 32 h exhibited bright AIF staining that clearly included the nuclei (indicated by counterstaining with DAPI), suggesting that AIF had completely translocated to the nucleus by this time. Therefore, nuclear translocation of AIF clearly followed activation of Bax and coincided with the manifestation of an apoptotic morphology. Conformational activation of Bax and nuclear translocation of AIF also was observed in HSF4012 cells treated with the <3-kDa fraction of the apoptotic conditioned medium (data not shown). Taken together, these results suggest that a toxic factor in the apoptotic medium induces activation of Bax and nuclear translocation of AIF. Since Bax

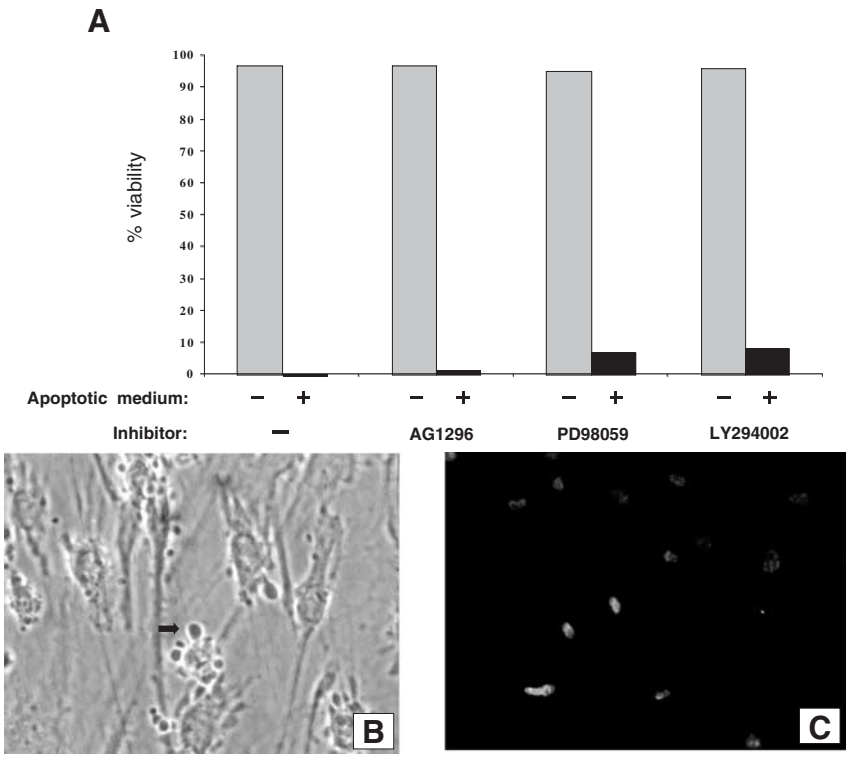


**FIGURE 9.** The effect of conditioned medium from dead E5-transformed HDFs on normal untransformed HDFs. **A.** Subconfluent untransformed HSF4012 cells were either untreated (−) or were pretreated with the kinase inhibitors AG1296, LY294002, or PD98059 by addition of the inhibitor directly to the medium at final concentrations of 17.5, 20, or 50 μM, respectively. Two days later, after the cells had reached confluence, the cells were either left under these conditions (−) or had their medium replaced with medium from apoptotic E5-transformed HDFs (+) in the presence or absence of the respective inhibitor. Two days later, when the medium-treated cells appeared to be undergoing apoptosis, the cell viability was determined by a trypan blue dye exclusion assay. Phase-contrast and TUNEL-stained images of HSF4012 cells treated with the apoptotic medium in the absence of any inhibitors are shown in **B** and **C**, respectively. Images of cells were captured and analyzed as described in Fig. 6. The *arrow* in panel **B** points to membrane blebbing, which is characteristic of apoptotic cells.

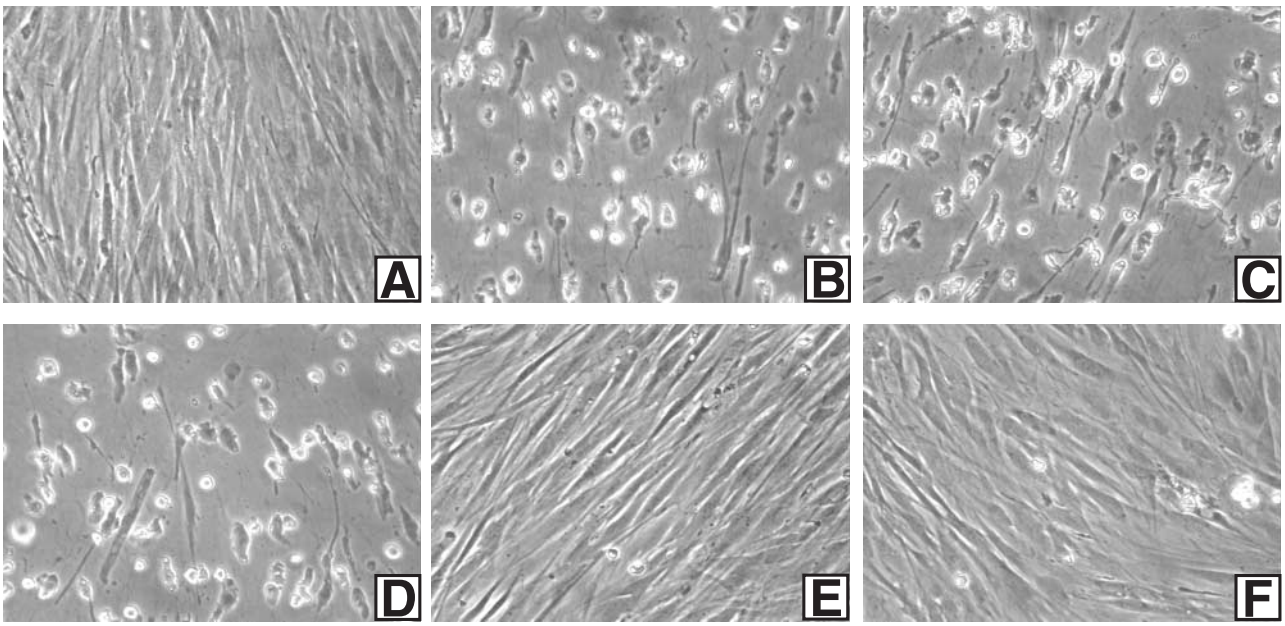


**FIGURE 10.** Partial characterization of a toxic factor in the conditioned medium of apoptotic E5-transformed HDFs. Confluent untransformed normal HSF4012 cells were either untreated (**A**), or had their medium replaced with conditioned medium from dead E5-transformed HDFs that was either untreated (**B**) or subjected to various pretreatments. Pretreatments included boiling (**C**), removal of high molecular weight solutes by centrifugation through a filter device with a molecular weight cut off pore size of 3 kDa (**D**), and incubation with proteinase k (**E** and **F**). Proteinase k was inactivated in the medium by boiling (**E**) or removed from the conditioned medium by filtration through the 3-kDa cut off centrifugal filter device (**F**) before medium replacement. Photomicrographs were taken 2–3 days after media replacement as described for Fig. 8.

activation occurred relatively early and preceded nuclear translocation of AIF by at least 24 h, it is possible that the toxic factor directly stimulates activation of Bax, which in turn results in mitochondrial dysfunction followed by relocalization of AIF and apoptosis.

## Discussion

In this paper, we demonstrated that mortal HDFs transformed by the BPV E5 oncoprotein died via apoptosis 2 weeks after they reached confluence. Similar results were observed with HDFs expressing *v-sis*, the viral homologue of PDGF BB. We also provide evidence that apoptosis of the transformed HDFs occurs through a caspase-independent, Bcl-2-resistant pathway that may involve mitochondrial dysfunction and nuclear translocation of AIF. Furthermore, our data indicate that a small toxic peptide secreted by the transformed cells induces this apoptosis perhaps through conformational activation of the proapoptotic protein Bax.

Mitochondrial dysfunction is likely to be the primary basis for apoptosis of the E5-transformed HDFs. We showed that the mitochondrial proteins cytochrome *c* and AIF were released into the cytosol of these cells before death, with a redistribution of cytochrome *c* in the cytoplasm and nuclear translocation of AIF. Accumulated evidence suggests that cytochrome *c* and AIF release lead to caspase-dependent and caspase-independent apoptotic pathways, respectively (25–27). Therefore, cytochrome *c* release may account for the caspase 3 activation observed in the apoptotic cells. However, because caspase inhibitors did not prevent apoptosis, a caspase-dependent pathway is not likely to play a major role in apoptosis of these cells. Instead, a caspase-independent pathway triggered by AIF release and its translocation to the nucleus could be delivering the crucial insult leading to cell death. Nonetheless, it is still possible that cytosolic cytochrome *c* may be predisposing the cells to apoptosis through some unknown caspase-independent mechanism.

Proapoptotic Bcl-2 family members are thought to promote cytochrome *c* and/or AIF release by increasing the mitochondrial membrane permeability. Some proapoptotic proteins, such as Bax, can enhance the mitochondrial membrane permeability independent of their ability to heterodimerize and antagonize the anti-apoptotic Bcl-2-like proteins (32). Upon an apoptotic stimulus, Bax is thought to undergo a conformational change, homodimerize, translocate to the mitochondria, and either insert directly into the mitochondrial membrane to form a channel or interact with a preexisting pore complex (28, 29, 32–35). Bax is a logical candidate for mediating mitochondrial dysfunction in the transformed HDFs because an activating conformational change in Bax was observed before death. Moreover, heterologous overexpression of Bax was shown in previous studies to result in caspase-independent apoptosis while also inducing caspase activation and cytochrome *c* release from the mitochondria (35–38), a situation that was also observed for the E5-transformed HDFs. Other studies showed that a cleavage product of the proapoptotic protein Bid associates with and promotes a conformational change in Bax, resulting in its activation (29, 32, 33). However, such a Bid cleavage product could not be detected in the E5-transformed cells (data not shown), suggesting that Bid may not be responsible for Bax activation in these cells. Given the rapid kinetics of Bax activation in response to treatment with conditioned medium from apoptotic E5-transformed cells (Fig. 11), the activating conformational change in Bax instead

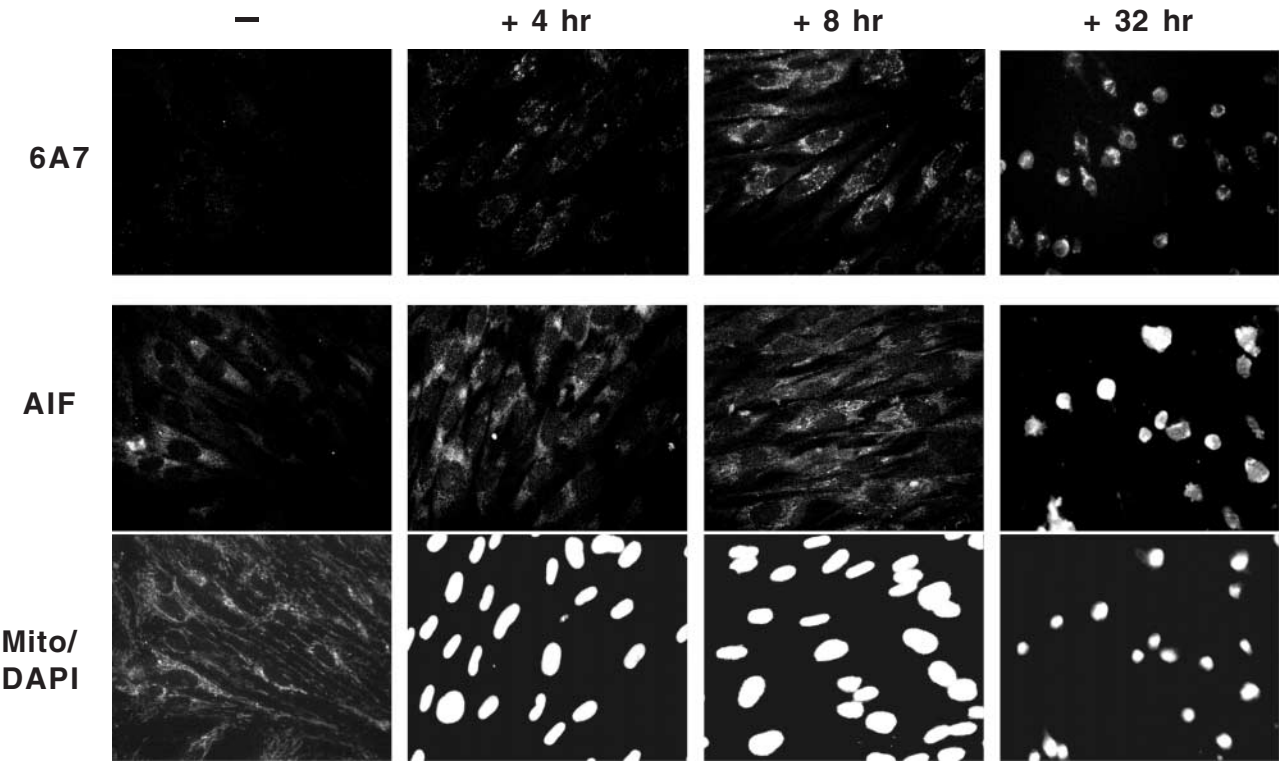


**FIGURE 11.** Immunofluorescence staining for active Bax and AIF in HDFs treated with the apoptosis-inducing conditioned medium. Normal HSF4012 cells were either untreated (–) or had their medium replaced (+) with conditioned medium from apoptotic E5-transformed HDFs. Four, 8, and 32 h later, the cells were fixed and stained with the conformation-specific anti-Bax antibody (*6A7*), or anti-AIF antibodies. Counterstaining of the AIF-stained cells with Mitotracker or DAPI was performed to visualize mitochondria or nuclei, respectively (*bottom panels*). Images of cells were captured using a digital camera interfaced with a fluorescence microscope and analyzed as in Fig. 6.

may be induced by a toxic factor produced by the transformed HDFs. As discussed below, it is possible that the toxic peptide may directly interact with Bax to affect this conformational change.

The proapoptotic protein Bcl-$X_S$ also could be facilitating mitochondrial dysfunction of the E5-transformed HDFs because an increase in its expression was observed before cytochrome *c* release into the cytosol. Bcl-$X_S$ is a smaller isoform of Bcl-$X_L$ (39), and little is known of the mechanism by which it induces apoptosis. One theory is that Bcl-$X_S$ may function to sensitize actively proliferating or transformed cells to undergo apoptosis. This is based on the finding that overexpression of Bcl-$X_S$ selectively induced apoptosis in c-*myc*- and *erb*B2-transformed mammary epithelial cells compared to untransformed cells (40). In addition, treatment of vascular smooth muscle cells with PDGF BB was found to induce proliferation as well as Bcl-$X_S$ expression and apoptosis (41). Together, these studies raise the possibility that activation of PDGFβR signaling pathways in the E5-transformed HDFs leads to increased Bcl-$X_S$ expression, which in turn may contribute to mitochondrial dysfunction in these cells.

Despite evidence for mitochondrial dysfunction in the E5-transformed HDFs, overexpression of Bcl-2, a well-established guardian of mitochondrial function, did not protect the cells from apoptosis. Moreover, apoptosis of these cells occurred despite a substantial elevation in Bcl-2 levels between confluence and death. This increase in Bcl-2 expression could represent an ineffective protective response to mitochondrial dysfunction. We also found that enforced overexpression of Bcl-2 in the normal HDFs protected against staurosporine-induced apoptosis but not against death induced by the apoptotic medium from the transformed HDFs. Thus, although the exogenously expressed Bcl-2 was functional in this system, it was ineffective in response to a toxic substance in the apoptotic medium. This may be because the toxic factor either inhibits Bcl-2 and/or acts in a Bcl-2-independent manner (see below).

The toxic factor produced by the transformed HDFs appears to be a peptide that is less than 3 kDa in size. Indeed, if this peptide was less than 1 kDa, as our preliminary observation suggests, it would probably consist of only 8–10 amino acids. Several small naturally occurring peptides have been reported to induce apoptosis. For example, Buckley *et al.* (42), demonstrated that short peptides containing the integrin-recognition RGD motif were able to enter cells and induce apoptosis independent of integrin-mediated cell clustering by directly affecting autoprocessing and activation of caspase 3. Because caspase 3 activity was required for apoptosis induced in this manner, it is unlikely that the peptide released by the E5-transformed HDFs is an RGD-containing peptide. Another peptide reported to induce apoptosis is the octapeptide angiotensin II (43, 44). However, it is unlikely that this is the toxic peptide produced by the HDFs, because angiotensin II treatment of these cells did not induce apoptosis (data not shown). Finally, a 10-amino acid fragment of the amyloid-β peptide, which is associated with neuronal degeneration in Alzheimer's disease, was found to induce apoptosis in neuronal cells (45, 46). Neuronal cell death induced

by the amyloid-β peptide was shown to occur through a Bax-dependent, caspase-independent mechanism that involved mitochondrial dysfunction (46, 47), and hence may be similar to apoptosis of the E5-transformed HDFs. Furthermore, one report showed that apoptosis induced by the amyloid-β peptide required a chronic exposure to the peptide (45). This is similar to our observation that sustained treatment with the apoptotic conditioned medium is required for cell death. Therefore, it is possible that the toxic factor produced by the transformed HDFs shares properties with the amyloid-β peptide.

There are several ways in which the putative toxic peptide produced by the transformed HDFs could induce apoptosis. First, the peptide could bind to and inhibit a survival factor in the medium. However, preliminary results showing that the toxic activity in the medium can be depleted by incubation with cells (data not shown) argue against this possibility and suggest instead that the peptide binds to and/or enters the cells. For instance, the peptide could bind to a receptor on the cell surface and induce a signaling cascade resulting in apoptosis. Alternatively, if the peptide is cell permeable, it could enter the cell and induce apoptosis by increasing the mitochondrial membrane permeability. The fact that sustained treatment with the toxic peptide is required for inducing apoptosis suggests that accumulation of the peptide within a particular cellular compartment such as the mitochondria may be required for commitment to apoptosis. Our evidence suggests that the toxic peptide may directly induce an activating conformational change in Bax. This implies that the peptide could bind to Bax and thereby facilitate its activation and insertion into the mitochondrial membrane. If this peptide binds to Bax, theoretically, it could affect a conformational change in Bax, form a pore-like structure, and at the same time block Bcl-2 binding. In this way, the toxic peptide could facilitate the release of cytochrome *c* and AIF in a Bcl-2-resistant manner. If the toxic peptide binds to Bax, it could be structurally similar to the Bcl-2 homology 3 (BH3) domain of the proapoptotic Bcl-2 proteins, because this domain is responsible for hetero/homodimerization (48) as well as the apoptotic activity of these proteins (32). Indeed, several studies have shown that synthetic peptides derived from Bcl-2 homology 3 domain of proapoptotic Bcl-2 proteins induce apoptosis through competitive inhibition of anti-apoptotic Bcl-2 proteins as well as by directly inserting into the mitochondrial membrane (49–52).

Because apoptosis of the E5-transformed HDFs is initiated consistently 13–14 days after confluence, we speculate that an event occurring at or near confluence primes the cells for apoptosis 2 weeks later. Furthermore, inhibition of the PDGFβR or PI 3-kinase activity before but not after confluence prevents apoptosis, suggesting that activation of certain signaling pathways before confluence most likely is crucial for apoptosis 2 weeks later. Because PDGFβR kinase activity was not required for the normal HDFs to undergo apoptosis in response to the toxic peptide, PDGFβR signaling most likely is required for production of the toxic peptide rather than the apoptotic process itself. Thus, we propose that production of the toxic peptide is initiated at or near confluence through a PDGFβR signaling pathway. Furthermore, our observation that the apoptosis-inducing activity in conditioned medium from the E5-transformed HDFs progressively increases as the cells approach death supports the theory that the toxic peptide gradually accumulates to toxic levels over time. The toxic peptide is probably not equivalent to the previously described factor secreted at confluence (13), which induces PDGFβR receptor degradation and growth inhibition, because the latter is insensitive to proteinase k treatment.[1] Nonetheless, the factor secreted at confluence might trigger a pathway that eventuates in secretion of the apoptosis-inducing peptide. Subsequently, this peptide could affect an activating conformational change in Bax, which then would increase the mitochondrial membrane permeability in a Bcl-2-resistant manner. Accumulation of the peptide over a period of 2 weeks could lead to the release of AIF from the mitochondria and its subsequent translocation to the nucleus where it would deliver the final insult leading to cell death.

In conclusion, we describe a novel mode of apoptosis as a consequence of partial transformation of mortal human fibroblasts by the BPV E5 oncoprotein. This apoptotic mechanism may be activated in mortal human cells as a natural protective response to certain aspects of transformation. Identifying the pathway leading to production of a toxic peptide by these cells should provide a mechanistic relationship between transformation and apoptosis. Moreover, once identified, the toxic peptide itself could be of therapeutic value, as it could be used to specifically kill cancer cells if linked to a tumor antigen-targeting moiety (53). Finally, characterization of the peptide's mode of action promises to reveal important new insights into mechanisms of apoptosis.

## Materials and Methods

### *Inhibitors and Antibodies*

The following inhibitors were purchased from the indicated companies: tyrphostin AG1296, SB203580, and apigenin, Calbiochem, San Diego, CA; LY294002, Biomol Research Laboratories, Plymouth Meeting, PA; PD98059, New England Biolabs, Beverly, MA; Ac-DEVD-CHO, PharMingen, San Diego, CA; ZVAD-fmk, Bachem, King of Prussia, PA. Antibodies against the following proteins were purchased from the companies indicated: Bax, Upstate Biotechnology, Lake Placid, NY; OxPhos Complex (Cox) IV subunit II (human), Molecular Probes, Eugene, OR; Bcl-2, Biosource International, Camarillo, CA; actin, Sigma. Antibodies against AIF and the conformationally active form of Bax (antibody 6A7) were purchased from Exalpha Biologicals, Boston, MA. Antibodies against Bcl-X (which recognizes both isoforms Bcl-$X_L$ and Bcl-$X_S$), cytochrome *c*, caspase 3, and PARP were purchased from PharMingen. Antibodies against Bid, Rb, and p53 were purchased from Santa Cruz, Santa Cruz, CA. Rabbit polyclonal anti-PDGF receptor antiserum, which recognizes the COOH-terminal 13 amino acids of the human PDGFβR, was a gift from Dr. Daniel DiMaio (Yale University). Mitotracker dye was purchased from Molecular Probes.

### *Cell Culture*

HSF4012 (NHDF4012) human foreskin fibroblasts (HDFs) were purchased from Clonetics at passage 1 and maintained in α-MEM (Life Technologies, Inc., Grand Island, NY) supple-

[1] L. M. Petti, unpublished observation.

mented with 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin (α-MEM-10). Cell strains stably expressing the BPV E5 gene were established by retroviral mediated gene transfer as previously described (13). Control cells were established by infection with the corresponding retrovirus without an insert. MCF-7 and MDA cells were also maintained in α-MEM-10. C127, 293, and SK-N-SH cell lines were maintained in DMEM supplemented with 10% FBS and antibiotics.

### *Trypan Blue Dye Exclusion Assay*

E5-expressing HDFs were seeded into multiple 60-mm dishes at $10^6$ cells/dish. Each day between 11 and 15 days after the cells reached confluence, cells in triplicate dishes were trypsinized, pelleted, washed with PBS, resuspended in a solution of 0.2% trypan blue (Life Technologies, Inc.), and counted using a hemacytometer. Both live (dye excluded) and dead (stained) cells were counted, and percentage viability was calculated. The results from triplicate dishes were used to determine the mean percentage viability and SD for each time point.

### *TUNEL Analysis*

A TUNEL assay was performed using TdT and fluorescein-dUTP purchased from Boehringer Mannheim, Mannheim, Germany. Live or dead HDFs grown on coverslips in 60-mm dishes were fixed with 4% paraformaldehyde in PBS (pH, 7.4), permeabilized in a solution of 0.1% Triton X-100 in 0.1% sodium citrate, and incubated with a 1:1 mixture of TdT and fluorescein-dUTP as described by the manufacturer. Cells were then counterstained for cellular DNA with 2 μg/ml DAPI in PBS for 5 min and visualized by fluorescence microscopy using a Zeiss fluorescent microscope. Images were captured using a digital camera interfaced with the microscope and analyzed using the Scion Image (Scion Corp.) analysis program. Dead cells incubated with fluorescein-dUTP alone were used to determine nonspecific background staining.

### *Time-Lapse Video Microscopy*

Time-lapse video microscopy was carried out using a Nikon inverted microscope fitted with phase-contrast optics and time-lapse phase-contrast images were captured with a video camera as described (54). Temperature was maintained at 37°C in a Red Beam Incubation Opti-Quip system (Highland Hills, NY). Individually time-stamped images were taped sequentially at 12-s intervals using a video recorder. Taping of apoptotic cells was initiated when the cells first displayed morphological signs of death and continued for 48 h thereafter.

### *Immunoblot Analysis*

To prepare whole cell extracts, cell monolayers in 60-mm dishes were washed twice with PBS and then lysed by adding 200–250 μl of hot 2× Laemmli sample buffer. Cell extracts were then passed through a 25-gauge needle, boiled, and subjected to SDS-PAGE. The following polyacrylamide concentrations were used: 7.5% (for PDGFβR, PARP, and AIF blotting), 12% (for caspase 3 blotting), 15% (for Bax, Bcl-2, cytochrome *c*, and Cox IV, subunit II blotting). Gels were transferred to nitrocellulose (for PDGFβR blots) or PVDF (for all other blots) at 100 V for 2 h or at 30 V overnight. Immunoblotting was performed as previously described (13) using 5% nonfat dry milk as blocking agent and a 1:500–1:1000 dilution of antibody. Proteins were detected by enhanced chemiluminescence (Amersham, Piscataway, NJ) using a protein A- or anti-mouse IgG-horseradish peroxidase conjugate (Pierce, Rockford, IL).

### *Subcellular Fractionation*

Cytosolic and mitochondrial fractions were prepared as previously described (55) with some modifications. Briefly, HDFs in a 60-mm dish were washed twice in cold PBS, scraped in 300 μl of isotonic buffer A (20 mM HEPES-KOH, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 15 μg/ml leupeptin, 15 μg/ml aprotinin), and incubated on ice for 30 min. Cells were homogenized using a glass dounce homogenizer, transferred to a microcentrifuge tube, and nuclei and unbroken cells were pelleted by centrifugation at 750 × *g* for 10 min at 4°C. The supernatant fraction was then centrifuged at 10,000 × *g* for 15 min at 4°C, and the resulting pellet was considered the mitochondrial fraction. The supernatant from this step was further centrifuged at 21,000 × *g* for 30 min at 4°C, and the resulting supernatant was considered the cytosolic fraction. After determining the protein concentration of the cytosolic fraction using a Coomassie protein assay reagent (Pierce), 20–30 μg were mixed with an equal volume of 2× Laemmli sample buffer and then subjected to immunoblot analysis.

### *Immunofluorescence Staining*

Cells grown on coverslips were washed twice with PBS and then fixed with a 1:1 solution of methanol:acetone. Fixed cells were permeabilized by freeze-thawing, washed with PBS, and then blocked in 3% BSA/PBS for 30 min at room temperature. The cells were then incubated with a 1:50 dilution of primary antibody (antibody 6A7, which recognizes the conformationally active form of Bax, anti-cytochrome *c* antibody, or anti-AIF antibody) for 3 h at 37°C in a humidified chamber. After three to five washes with PBS, the cells were incubated with a 1:250 dilution of Alexa Flour 488-anti-rabbit IgG (Molecular Probes) or fluorescein-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. For AIF staining, cells were washed and then counterstained for cellular DNA with 2 μg/ml DAPI in PBS for 5 min. Cells were washed as above and mounted in 20% glycerol/PBS. To visualize mitochondria, live cells were incubated with medium containing 20 nM Mitotracker dye (Molecular Probes) for 30 min at 37°C before fixing the cells for immunofluorescence. Images were captured using a digital camera interfaced with a Zeiss fluorescent microscope and analyzed using the Scion Image program (Scion Corp.).

### *Heterologous Overexpression of Bcl-2*

A human Bcl-2 cDNA clone (gift from Dr. Dana Crawford, Albany Medical College) was subcloned into the LXSN retroviral vector, which carries the G418 resistance gene, by standard methods, generating Bcl-2-LXSN. High titer amphotropic retrovirus was obtained as previously described (13) after

transient transfection of phoenix amphotropic producer cells (American Type Culture Collection, with permission of Dr. Gary Nolan, Stanford University) with 10 μg of Bcl-2-LXSN or the empty LXSN retroviral vector. To establish HDFs expressing E5 with or without exogenous Bcl-2, the E5-transformed HDFs were infected with high titer Bcl-2 or LXSN retrovirus and then selected for G418 resistance by culturing the cells in media containing 400 μg/ml of G418 as described previously (13).

### *Fractionation of Conditioned Medium*

Conditioned medium from dead E5-transformed HDFs was fractionated by centrifugation at 3000 × *g* for 3 h at room temperature through a Centriplus (Millipore, Bedford, MA) centrifugal filter device with a molecular weight cut off pore size of 3 kDa. The filtrate, which contained solutes smaller than 3 kDa, was used to replace the existing medium of normal HDFs. Conditioned medium from dead E5-transformed HDFs was also fractionated by dialysis using a Tube-O-Dialyzer (Geno Technology, Inc., St. Louis, MO) having a dialysis membrane molecular weight cut off pore size of 1 kDa. Dialysis was performed twice against a 25× volume of fresh α-MEM. The retentate in the dialysis tube, which contains solutes larger than 1 kDa, was used to replace the existing medium of normal HDFs.

### *Proteinase Treatment of Conditioned Medium*

Proteinase k (Boehringer Mannheim), trypsin (Sigma), chymotrypsin (Sigma), or endoproteinase Glu-C (Boehringer Mannheim) was added to conditioned medium from dead E5-transformed HDFs at a final concentration of 10, 200, 250, and 160 μg/ml, respectively. The conditioned medium was then incubated for 30 min at 37°C (for proteinase k and endoproteinase Glu-C) or 25°C (for trypsin and chymotrypsin). Inactivation of the proteinases was accomplished either by boiling the conditioned medium for 5 min or by centrifuging the medium through a Centriplus (Millipore) centrifugal filter device with a molecular weight cut off pore size of 3 kDa. The treated medium was then filtered through a 0.45-μm syringe filter before adding to cell monolayers.

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