Apoptosis Is Accompanied by Changes in Bcl-2 and Bax Expression, Induced by Loss of Attachment, and Inhibited by Specific Extracellular Matrix Proteins in Mammary Epithelial Cells

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Abstract
Mammary epithelial cells (MEC) undergo programmed cell death (PCD) when deprived of serum and growth factors at high cell density but not at low density. The addition of epidermal growth factor and insulin to serum-free medium (SFM) completely restores cell survival. In this report, we examine the role of cell-cell and cell-matrix interaction. When cell attachment is prevented, PCD is markedly accelerated. This effect is observed in cells collected at low or high density and is unaffected by calcium depletion. Cells plated in SFM on purified laminin, tenascin C, or collagen IV-coated dishes, as well as on dishes coated with endogenous extracellular matrix deposited by HC11 mammary cells, show reduced PCD. The addition of soluble laminin or tenascin C to suspension cultures of MECs also partially inhibits PCD. In contrast, no effect is seen with fibronectin or collagen I. These results indicate that reduced contact with a solid substrate contributes to the induction of PCD, which might partially explain the fact that it is only observed in confluent cultures. Ectopic Bcl-2 expression in MCF10-A and HC11 mammary cells results in a complete suppression of PCD. In MCF10-A cells, the level of endogenous Bcl-2 increases when the survival factors epidermal growth factor and insulin are added to the SFM but is unaffected by cell density. On the contrary, Bax protein expression increases sharply with cell density but does not change upon addition of epidermal growth factor and insulin. When compared to lactating tissue, Bcl-2 protein levels decrease during mammary gland involution. Bax protein levels increase during lactation and remain high during involution. These data suggest that Bcl-2 and Bax might be intracellular mediators of signals that influence MEC apoptosis.

Introduction
The mechanism and regulation of PCD in mammalian cells is receiving increasing attention. The rapid and efficient elimination of unwanted cells within an organism is at least as important for tissue function and homeostasis as the generation of new cells by cell proliferation (1). This regulation is particularly true for tissues with a renewing cell population like the epithelium of the digestive tract, or organs like the mammary gland undergoing cycles of growth, differentiation, and involution. In both instances, the cell number has to be accurately controlled (reviewed in Ref. 2). When the appropriate cell type-specific signals are received, a series of intracellular molecular events, only partly known, initiate and carry out a process of cell shrinkage, DNA fragmentation, and nuclear condensation also known as apoptosis (reviewed in Refs. 3 and 4).

The mammary gland provides a unique and ideal model system to study the signals that initiate apoptosis and the intracellular signal transduction pathways that are activated during the process. During postlactational involution, a large fraction of the MECs that compose the secretory part of the gland is eliminated by PCD (5, 6). In vivo, the ECM plays a critical role in apoptosis of MECs (5, 7–9). During involution, the basement membrane is rapidly degraded due to the proteolytic action of secreted enzymes including stromelysins, type IV collagenase, gelatinase, urokinase-type plasminogen activator, and tissue-type plasminogen activator (5, 7, 10–13).

The composition and structure of the ECM and the contact of MECs on their physiological substrate, the basement membrane, are known to have important signaling functions. In addition to providing mechanical support and the necessary information for cells to establish their polarity and morphology, signals derived from ECM are necessary for the expression of MEC-specific differentiation proteins, such as β-casein (14–16). Most of the data have been obtained from models of differentiating cultured MECs. Similar to differentiation, PCD can also be modeled and reproduced in vitro (9, 17, 18). Using these models, the importance of ECM in controlling apoptosis of MECs can be studied in more detail.

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3 The abbreviations used are: PCD, programmed cell death; MEC, mammary epithelial cell; ECM, extracellular matrix; BH, Bcl-2 homology; EGF, epidermal growth factor; NAN, number of apoptotic nuclei; SFM, serum-free medium; GM, growth medium; CFM, calcium-free medium; MoAb, monoclonal antibody; FBS, fetal bovine serum; PI, propidium iodide.
It has been shown that MECs undergo apoptosis when plated on plastic but not when plated on laminin or Matrigel, and that β1 integrin receptors mediate at least part of this survival signal (9, 19). Also related to these observations is the fact that loss of attachment to a solid substrate can induce apoptosis in some cell lines (20–23). However, the specific ECM component(s) involved, the intracellular molecule(s) that convey apoptotic or anti-apoptotic signals, as well as the contribution of cell–cell contact and growth factor depletion, remain to be clarified.

An important class of molecules that plays a role in regulating the rate of PCD is the Bcl-2 family of cytoplasmic proteins. The ability of Bcl-2 to suppress PCD is well documented (24–27), although the mechanism remains elusive. A number of related proteins that contain BH regions have been shown to either suppress (Bcl-X<sub>L</sub>) or promote (Bax, Bcl-X<sub>S</sub>, Bad, and Bak) PCD when overexpressed in various cellular systems (4, 28–32). These proteins appear to function by homo- and heterodimerization via the BH domains (33). It has been suggested that the ability of Bax to promote cell death is due to its capacity to heterodimerize with and inactivate Bcl-2 in a dominant fashion, or alternatively, Bax homodimers might accelerate PCD on their own (28, 32, 34, 35). In the latter case, Bcl-2 and Bcl-X<sub>S</sub> could suppress apoptosis by heterodimerizing and inactivating Bax (32). A quantitative analysis indicated that when one-half or more of endogenous Bax is heterodimerized with Bcl-2 or Bcl-X<sub>S</sub>, apoptosis is repressed (32), indicating that the relative levels of these BH-containing proteins determine the fate of the cell (36). The ability of a Bcl-2-related protein to promote or suppress apoptosis has been assessed in transfected cells (4, 26–32). Because of the ever-increasing number of Bcl-2-related proteins and the numerous stimuli that induce apoptosis, less is known about the role that a particular Bcl-2 family member has in a natural setting.

We have shown previously that high density cultures of nontransformed MECs undergo PCD when deprived of serum and growth factors, and that this effect requires high cell density. Under these conditions, the addition of insulin and EGF can efficiently restore cell survival (18). Thus, in conjunction with growth factor starvation, cell density represents a signal that promotes apoptosis of MECs. Is this report, we asked the question whether the effect of cell density is due to increased cell–cell contact or reduced contact to ECM? The results show that loss of contact, rather than cell–cell adhesion, is a potent activator of PCD of MECs. Contact with specific ECM proteins, including laminin, tenascin C, and collagen type IV, partially restores survival. We determined the levels of Bcl-2 and Bax proteins in these cells under apoptotic and nonapoptotic conditions. Here we show that high cell density is accompanied by an increase in the Bax protein level, whereas Bcl-2 levels increase following the addition of survival factors. During lactation and involution, there is a decrease in the level of Bcl-2 and a concomitant increase in the level of Bax. These data indicate that in some situations, Bcl-2 and Bax might mediate extracellular apoptotic signals and regulate the rate of PCD in MECs.

Results

Loss of Attachment Accelerates Apoptosis. We have observed previously that confluent cultures of nontransformed MEC lines undergo PCD when maintained at high cell density in SFM. Little or no difference was observed if the cells were maintained subconfluent in SFM. The addition of EGF and insulin to the SFM reduces the NAN to basal levels (18). This response appears to be a general feature of mammalian epithelial cells.

We attempted to determine the molecular mechanisms underlying the requirement of high cell density for this apoptotic response. We speculated that it could be related to reduced attachment to a solid substrate, due to a more limited space, or to increased cell–cell contact. We also asked whether the apoptotic response is acquired by the cells at confluence or can be induced in all cases. To examine these questions, we used plates coated with poly-HEMA, a nontoxic hydrophobic substance that prevents cell attachment. MCF10-A and HC11 cells were plated on poly-HEMA-coated dishes in either GM, SFM, or SFM supplemented with EGF (10 ng/ml) and insulin (5 μg/ml) for a maximum of 24 h and then scored for NAN. As control, cultures were plated on plastic, grown to confluence, and switched to SFM for 48 h. As shown in Fig. 1, control cells displayed approximately 10–12% NAN. In contrast, cells plated on poly-HEMA-coated dishes displayed accelerated apoptosis. The MCF10-A and HC11 cells maintained in suspension for, respectively, 16 and 8 h in SFM, displayed levels of NAN similar or higher than those observed in confluent adherent cultures after 48 h in SFM (Fig. 1, A and B). After 24 h in SFM, a high rate of apoptosis was observed (40–50% NAN). Compared to cells in SFM alone, cells plated on poly-HEMA-coated dishes and incubated in GM or in SFM supplemented with EGF and insulin showed a substantial reduction in NAN. However, a residual 6–7% NAN is observed for both cell lines, even in the presence of survival factors. This is significantly higher than the 0.5–1% NAN observed for adherent cells grown in the same conditions. In conclusion, loss of attachment accelerates apoptosis of MECs under all growth conditions, and EGF and insulin are always able to restore survival.

DNA fragmentation analysis was carried out on DNA samples from MCF10-A and HC11 cells maintained in suspension for 16–24 h in either GM or in SFM. A nucleosomal DNA ladder typical of apoptotic cells was observed in cells in SFM but not in GM (Fig. 1C). These results further confirm the apoptotic nature of the cell death induced by loss of attachment in SFM.

Cells from Low and High Density Cultures Respond Equally to Loss of Attachment. We then asked whether HC11 and MCF10-A cells in high density cultures acquire an apoptotic response de novo, or if this response can also be induced in cells at low density. MCF10-A and HC11 cells were collected from sparse or dense cultures and then plated on poly HEMA-coated dishes in SFM and scored for the NAN. HC11 cells (Fig. 2) and MCF 10-A cells (data not

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4 G. R. Merlo, unpublished data.
A. MCF10-A

![Graph showing apoptosis levels for MCF10-A cells on plastic and poly-HEMA.](image)

B. HC11

![Graph showing apoptosis levels for HC11 cells on plastic and poly-HEMA.](image)

C. MCF10-A and HC11 cells

![Images of MCF10-A and HC11 cells maintained confluent in GM or SFM.](image)

**Fig. 1.** Loss of attachment and apoptosis of MECs. Shown are MCF10-A cells (A) and HC11 cells (B) maintained in GM (√), SFM (□), or in SFM + insulin and EGF (▲). Cells were plated on plastic and maintained confluent for 48 h (left part of each diagram) or plated on poly-HEMA-coated dishes (right part) for 8, 16, or 24 h, as indicated. NAN, the number of apoptotic nuclei over the total number of nuclei counted, in a percentage. C, DNA fragmentation in samples from MCF10-A (left) and HC11 (right) cells maintained confluent in GM or SFM. The typical apoptotic DNA fragmentation is observed when cells are maintained in SFM.

Shown) showed the same results. A similar level of apoptosis (40%) was observed for cells collected from low or high density. As control, cells were also re-plated on plastic at low density, in which case the NAN was less than 2% in both SFM and SFM + EGF and insulin. Survival was restored by the addition of EGF and insulin (9% NAN), and no difference was observed when cells were collected form sparse or confluent cultures. Thus, the apoptotic response of MECs to loss of contact does not depend on properties acquired by the cells at high density.

**The Apoptotic Response of MECs Is Independent of Calcium-mediated Cell-Cell Contact.** Extensive cell-cell contact takes place when cells are confluent, and this largely depends on Ca$^{2+}$-dependent intercellular contact (37). To test for the role of Ca$^{2+}$-dependent cell-cell interactions in the apoptotic response of MECs, experiments were carried out in CFM. For the MCF10-A and the HC11 cells, cell-cell adhesion appears to be mainly Ca$^{2+}$-dependent, because in CFM, these cells remained mostly as a single-cell suspension when plated on poly-HEMA. Adherent confluent cultures of HC11 cells (Fig. 3) and MCF10-A cells (data not shown) were switched to CFM SFM for 48 h, in the presence or absence of the survival factors EGF and insulin. No difference was observed in the NAN as compared to a regular Ca$^{2+}$-containing SFM or to a CFM SFM to which CaCl$_2$ was added to a final concentration of 2 mM (Fig. 3A). Experiments with cells plated on poly-HEMA also showed no difference in the apoptotic response to loss of attachment, between cells maintained in regular SFM, in CFM SFM, or in CFM SFM supplemented with 2 mM CaCl$_2$ (Fig. 3, B and C). These results indicate that Ca$^{2+}$-dependent cell-cell adhesion does not play a major role in the apoptotic response of MECs.

**Immobilized or Soluble ECM Proteins Restore Survival of MECs.** We then tested the possibility that contact with ECM could have an apoptotic suppressing function in MECs. To do this, we used two independent strategies:

(a) Cells were plated at a high density (2 x 10$^6$ cells/well) on six-well cluster dishes coated with purified ECM proteins. Because of the high initial density, the cells were immediately confluent. Cells were then washed with PBS and switched to SFM for 12–16 h and scored for the NAN. A reduction in NAN was observed in the HC11 cells plated on collagen type IV (4.3%), on tenascin C (6.1%), and on laminin (9.3%) relative to control cells re-plated on plastic (16%). For the MCF10-A cells, a significant reduction relative to plastic (24%) was only observed when cells were plated on tenascin C (12.3%) or on laminin (15.6%) but not collagen type IV (24%). For both cell lines, collagen type I and fibronectin had no effect (Fig. 4A). Next, MCF 10-A and HC11 cells were plated at high or low cell density on dishes on which confluent HC11 cells were allowed previously to deposit their ECM and then removed, as described (38). A clear reduction in the NAN was observed when cells were confluent on ECM-coated dishes, compared to control cells plated on plastic (Table 1). This observation agrees with the fact that cells re-plated on plastic show higher NAN than cells allowed to grow and deposit their ECM (compare Figs. 1 and 4). Interestingly, the recovery by complete ECM was not better than that observed with individual ECM.
components. Finally, cells plated sparsely showed low NAN in all cases, as expected (Table 1 and data not shown).

(b) As a second strategy, we used the poly-HEMA assay described above and tested whether purified soluble ECM proteins added to the SFM could act as survival factors for cells in suspension. An initial experiment was carried out using purified laminin, fibronectin, tenascin C, and collagen type I added to the SFM at a concentration of 10 μg/ml. After the appropriate time (24 h for MFC10-A, 8 h for HC11), the NAN were scored. Laminin enhanced the survival of both cell lines, whereas tenascin C promoted survival of HC11 cells better than the MCF10-A cells (data not shown). The experiment was repeated using only laminin and tenascin C at various concentrations (0.5, 2, 5, and 10 μg/ml). The results demonstrate that laminin can partially restore survival of both MCF10-A and HC11 cells in the absence of attachment at concentrations as low as 0.5 μg/ml (Fig. 4B). For MCF10-A and HC11 cells, we observed a 70 and a 58% inhibition, respectively, of PCD (Fig. 4B). Tenascin C had a stronger effect on the HC11 cells than on the MCF10-A cells (Fig. 4B).

**Ectopic Bcl-2 Expression in MECs Suppresses Apoptosis.** The MCF10-A and the HC11 cells were infected with the MV12-mBcl2 retroviral vector and the corresponding MV12 vector as a control. Hygromycin-resistant MCF10-A cells were tested for ectopic Bcl-2 expression by SDS-PAGE, using the 124 MoAb that reacts with Bcl-2 protein of both human and murine origin (Fig. 5B). The Mf, 25,000 mBcl2 protein was observed only in the MV12-Bcl2 infected cells but not in the control infected cells or uninfected MCF10-A cells. The endogenous hBcl2 protein (p26) was detected in all samples. Because of a difference in affinity of this antibody to murine and human Bcl-2, it is not possible to estimate the relative level of expression. Expression of exogenous Bcl-2 in the infected cells was also confirmed using the 4C11 antibody, which specifically detects murine Bcl-2 (data not shown).

The infected cells were grown to confluency and either maintained in GM or switched to SFM for 24–48 h (Fig. 5). Uninfected and control infected HC11 and MCF10-A cells in SFM showed similar high levels of NAN, compared to cells in GM (Fig. 5, A and C). In contrast, the MV12-mBcl2 infected MCF10-A cells (Fig. 5A) and HC11 cells (Fig. 5C) failed to respond to this apoptosis-inducing treatment, and the NAN remained the same in GM or in SFM. Thus, expression of mBcl-2 efficiently blocks PCD in both cell lines.

**Bcl-2 and Bax Protein Expression in MECs.** Expression of endogenous Bcl-2 and Bax proteins in the MCF10-A cells was determined in total protein lysates by SDS-PAGE and immunoblotting with specific antibodies. No changes were observed in p26 Bcl-2 expression when samples from sparse or confluent MCF10-A cells were compared (Fig. 6A, top). This is true whether cells were kept in SFM or SFM supplemented with insulin and EGF. In contrast, the level of Bcl-2 protein in cells maintained in SFM plus insulin and EGF was 2.2-fold higher than cells in SFM (Fig. 6A). Thus, the level of Bcl-2 is increased in the presence of the survival factors but is not affected by cell density. The effects of insulin and EGF on Bcl-2 expression were also examined individually. The addition of insulin or EGF led to a 2.3- and a 1.3-fold increase in Bcl-2 level, respectively, after 24 h of treatment (data not shown).

Bax protein expression was analyzed in the MCF10-A, HC11, and NOG-8 MEC lines in extracts prepared from cells collected at various densities. In all three cell lines, higher Bax levels were observed in cells from high density cultures compared to low density cultures (Fig. 6 and data not shown). The most dramatic difference was seen in MCF10-A cultures grown in insulin and EGF, where there was 7-fold more Bax protein in cells from confluent cultures (Fig. 6A). Bax expression was also examined in MCF10-A cells plated on tenascin C, laminin, or plastic dishes (Fig. 6B). Although Bax expression was always higher in confluent cultures, there was no effect of the survival promoting ECM proteins tenascin C or laminin on Bax protein levels. Surprisingly, MCF10-A cells maintained for 16 h on poly-HEMA-coated plates, a condition which promotes apoptosis, expressed only low levels of Bax (Fig. 6B).

**Bcl-2 and Bax Protein Expression in the Mammary Gland.** High levels of apoptosis are observed during post-lactational regression of the mammary gland. To determine whether Bcl-2 or Bax may play a role in this process, the levels of the two proteins were examined during involution.
and compared to other stages of mammary gland development. The results of a Western analysis show that the Bcl-2 protein was present in glands from resting, virgin, and lactating animals. Two days following removal of the pups, there was a drop in Bcl-2, and at 3 days postlactation, Bcl-2 could not be detected (Fig. 7, top panel). A previous publication (39) showed that the Bcl-2 level dropped in late pregnancy; however, involuting tissue was not examined in this study, making a direct comparison to our results difficult. Bax protein, unlike Bcl-2, was nearly undetectable before and during pregnancy, and it increased at lactation and remained high during involution. In the murine mammary gland, histological signs of apoptosis can be seen beginning at day 2 through 4 days following removal of the pups (5, 6). Thus, at the time when the mammary gland shows morphological signs of PCD, the Bcl-2 protein is low and Bax is high. These results are consistent with their function as apoptosis suppressor and apoptosis promoter, respectively.

Discussion

We have shown previously that cell confluency together with growth factor depletion induce apoptosis of MECs (18). The results presented here suggest a possible mechanism underlying these observations. Namely, cells in high density cultures are more susceptible to growth factor starvation-dependent apoptosis due to reduced contact with a solid substrate. Furthermore, we show that the laminin, tenascin C, and collagen type IV, as well as the complete ECM, partially restore survival of these cells. These proteins were able to partially protect cells from apoptosis also in the absence of contact, suggesting that the effect is not directly related to the capacity of these molecules to promote adhesion to a solid substrate.

Histological observations of involuting mammary glands have revealed that extensive tissue remodeling and apoptosis is accompanied by degradation of the basement membrane (5, 40). Indications that loss of cell-matrix interaction is
Fig. 4. Inhibition of apoptosis of MECs by purified ECM proteins. A, MCF10-A cells (top panel) and HC11 cells (bottom panel) plated at high density on ECM-coated dishes and maintained for 24 h in SFM. Results with control uncoated dishes (plastic) are also shown. CG-I, collagen type I; CG-IV, collagen type IV; FN, fibronectin; TN, tenascin C; LM, laminin. B, MCF10-A (top panels) and HC11 cells (bottom panels) plated on poly-HEMA-coated dishes and maintained for, respectively, 24 and 8 h in SFM. Purified laminin (left panels) or tenascin C (right panels) were added to the SFM, at the indicated final concentrations. Cells were also maintained in SFM + insulin and EGF, as control (last column of the right panels). NANC, the number of apoptotic nuclei over the total number of nuclei counted, in a percentage.

<table>
<thead>
<tr>
<th>Cell density</th>
<th>Plated on:</th>
<th>Plastic</th>
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<tr>
<td>High (confluent)</td>
<td>17.0 ± 2.0c</td>
<td>12.5 ± 1.3</td>
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<tr>
<td>Low (sparse)</td>
<td>0.8 ± 0.2</td>
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*These data were obtained with HC11 cells. MCF 10-A cells showed similar results.

b ECM deposited by confluent HC11 cells in GM, according to Chammas et al. (36).

c Number of apoptotic nuclei over the total number of nuclei counted, in a percentage.

Table 1 Effect of ECM on apoptosis of HC11 cells*

an important apoptotic regulatory signal in MECs come from other types of studies. A number of proteases, including stromelysins, urokinase-type plasminogen activator, tissue-type plasminogen activator, collagenases, and gelatinases, are expressed during mammary gland involution, leading to basement membrane degradation (5, 7, 10–12). Interestingly, the tissue inhibitor of metalloproteinase mRNA, which encodes a metallo-proteases inhibitor, is transiently up-regulated early during involution, and tissue inhibitor of metalloproteinase pellet implantation into mammary glands delays involution (5, 12).

These data indicate that a delicate balance between ECM-degrading proteases and their inhibitors is responsible for basement membrane degradation and tissue remodeling. Ectopic expression of stromelysin-1 in cultured MECs and in transgenic mice resulted in a premature induction of apoptosis (8, 9, 41). In conclusion, a role for cell-matrix contact in apoptosis is clearly established. Basement membrane degradation that occurs during involution shuts down milk protein synthesis and induces PCD, thus playing a key role in tissue reorganization and the restoration of the original quiescent state. Our data further support this notion. In addition, MECs appear to be specifically sensitive to contact with basement membrane-derived ECM proteins, including laminin, tenascin C, and collagen type IV, but not mesenchyma-derived ECM proteins, including collagen type I and fibronectin.

Loss of contact with specific ECM molecules has been recognized as a pro-apoptotic signal also in other non-mammary cells (20–23). Thus, as a general phenomenon, contact with a substrate stimulates cell survival by inhibiting an intrinsic cell death program, which is then executed when contact is lost. The ECM molecules that participate in this effect, as well as the cognate cell surface receptors, are likely
Fig. 5. Ectopic Bcl-2 expression suppresses apoptosis in MECs. MCF10-A cells (A) or HC11 cells (C) were infected with the control vector (MV12) or with the Bcl-2 expression vector (MV12-Bcl2). Results with parental (uninfected), control, and Bcl-2-infected cells are shown. Cells were maintained confluent in GM or in SFM for 24 h. NAN, the number of apoptotic nuclei over the total number of nuclei counted, in a percentage. B, Western blot analysis of total protein extracts from uninfected MCF10-A cells (−), cells infected with control MV12 vector, and with the MV12-Bcl2 vector. The endogenous Bcl-2 protein (h-Bcl2, p26) is observed in all samples. The murine Bcl-2 protein (m-Bcl2, p25), encoded by the MV12-mBcl2 vector, is only observed in the Bcl-2-infected cells.

Fig. 6. Bcl-2 and Bax expression in MECs. A, Bcl-2 and Bax proteins in MCF10-A cells. Protein extracts were made from sparse (sp.) or confluent (cn.) cultures maintained either in SFM (left) or in SFM + insulin and EGF (right). Top panels, Bcl-2 protein (p26). Bottom panels, Bax protein (p21). B, Bax expression in MCF10-A cells plated at low or high density (left and middle panels, respectively) on untreated dishes (plastic) or dishes coated with purified laminin or tenascin C. Right, cells plated on poly-HEMA-coated dishes. C, Bax expression in NOG-8 cells. The p21 Bax protein (arrow) was detected as above. Protein extracts were made from cells grown to 70 and 100% confluency, and cells were kept confluent for 48 h (100 + %), as indicated on top, and maintained either in SFM (left) or in SFM + insulin and EGF (right).

5 Unpublished observation.
Integrin receptors (45), in our experiments, cells maintained adhesion to the substrate when kept in CMF, indicating that integrin function is unlikely to be affected. In contrast to MECs, inhibition of intercellular contact induces apoptosis in colon carcinoma cells (46), suggesting that again there is cell type specificity in the extracellular apoptotic signals.

The introduction of a Bcl-2 expression vector into MECs suppressed the PCD induced by confluence and growth factor withdrawal. In light of the fact that Bcl-2 appears to block PCD in almost all cases (26, 27), these results are not surprising. Ectopic overexpression of Bcl-2-related protein does not provide information about their in vivo function. It is worthwhile, therefore, to establish links between endogenous expression of Bcl-2-related proteins and extracellular apoptotic stimuli. The fact that there are many family members makes it difficult to assign a protective function or a death-promoting role to an individual protein. However, there are few examples where up-regulation of Bcl-2 or Bcl-X, is correlated with survival and an increase in Bax precedes cell death (25, 47–49). Interleukin 6 treatment, which causes survival of myocardial cells, leads to an up-regulation of Bcl-X (25). Bax levels increase in lymphoma cells preceding their apoptosis (50).

Bcl-2 family members are expressed in mammary tissue (50–53). Bcl-2 has been detected in nonlactating breasts of premenopausal women (51), and Bax has been observed in lactating murine mammary glands (52). In this report, we show that the expression of Bcl-2 and Bax varies, depending upon the differentiation status of the gland. Bcl-2 is expressed throughout pregnancy and lactation and drops at involution, whereas Bax is undetectable until lactation, when it rises and remains high at involution. The expression pattern of these two proteins in the mammary gland is consistent with the anti-apoptotic role of Bcl-2 and the pro-apoptotic role of Bax. Bax (54) and Bcl-2 (55–57)-deficient mice display specific defects in a limited number of organs, which likely reflects their tissue-specific roles in cell survival. Unfortunately, mammary gland development and involution has not yet been described in these knock-out mice.

The role of Bcl-2 and Bax in the apoptotic process initiated at confluence is less obvious. It is clear that Bax protein levels are higher in cells from confluent versus sparse cultures. However, confluent cultures plated on tenascin C and laminin, two ECM proteins which had a protective effect, displayed no decrease in the level of Bax expression. In addition, cells plated on poly-HEMA, a condition that promotes apoptosis, had the same level of Bax as sparse cells. It has been observed that primary MECs plated on protective or non-protective ECM had the same level of Bax protein. However, immunofluorescent studies revealed that Bax expression was very high in individual dying cells (39). In conclusion, it appears that the apoptosis induced in mammary cells by high confluence and serum-growth factor starvation is not strictly correlated with changes in Bcl-2 and Bax levels.

Materials and Methods

Cell Lines and Tissue Culture. The human breast epithelial cell line MCF10-A (58) was cultured in 1:1 DMEM/F12 GM supplemented with 10% FBS, EGF (10 ng/ml), insulin (5 μg/ml), and dexamethasone, as described (18). The HC11 murine mammary epithelial cells (59) were maintained in RPMI 1640 supplemented with 8% FBS, 10 ng/ml EGF, and 5 μg/ml insulin. The NOG-8 murine MECs (60) were cultured in DMEM supplemented with 10% FBS, 10 ng/ml EGF, and 5 μg/ml insulin. SFM was prepared from DMEM/F12 (for MCF10-A) or RPMI 1640 (for HC11) by the addition of 0.5 mg/ml fetuin, 10 μg/ml transferin, glutamine (2 mM), and gentamicin (10 μg/ml). SFM was prepared from RPMI 1640 or DMEM by omitting calcium from the formulation. Because serum also contains calcium, SFM was used serum free.

Induction and Detection of Apoptosis on Adherent Cells. Cells were plated on polystyrene chamber slides (LabTek) in GM, allowed to reach confluence, and then washed with PBS twice and incubated an additional 48 h in SFM, with or without 10 ng/ml EGF and 5 μg/ml insulin. The cells were then fixed with 2% formaldehyde for 10 min at 4°C, washed with cold PBS, and stained with a solution containing 50 μg/ml PI, 0.1% Triton X-100, 0.1% sodium citrate, and 20 μg/ml of RNase A in PBS for 15–20 min at room temperature. The apoptotic nuclei were counted by direct examination of their morphology using a Zeiss Axioskop fluorescence microscope equipped with a Neofluar ×40 objective lens. The results were expressed as a percentage of the NAN over the total number of nuclei examined. A minimum of 1000 nuclei were counted in each case.

Cell Culture and Detection of Apoptosis in the Absence of Attachment. Six-well cluster plates were coated twice with poly-HEMA (poly-hydroxyethyl-methacrylate) at a concentration of 10 mg/ml in 95% ethanol, dried, and washed three times with PBS. Cells were collected by trypsinization, centrifuged, and resuspended in the appropriate medium, then layered on the coated dishes at a density of 5 × 10^5 cells/ml and incubated at 37°C for the indicated time. Cells were then collected into microtubes at 4°C, centrifuged, fixed, and stained with PI as described above. The stained cells were then cytocentrifuged onto glass slides at 4°C (500 rpm for 5 min), air dried, and scored for NAN by fluorescence microscopy.

Protection of Apoptosis by ECM Proteins. The following purified soluble ECM proteins were used: laminin [from Engelbreth-Holm-Swarm tumors, purified according to Paulsson et al. (61)], tenascin C (from chicken embryo, prepared according to Chiquet-Ehrismann et al. (62)], fibronectin (from human serum; Boehringer) and collagen type I (from rat tail; GmbH & Co. Heidelberg, Germany). Six-well cluster plates coated with human fibronectin, rat collagen type I, and mouse collagen type IV were purchased from Collaborative Biomedical Products (Bedford, MA). Six-well plates coated with laminin and tenascin C were prepared by applying 1 ml of a 20 μg/ml solution of the purified proteins in PBS for 16 h at 4°C or for 4 h at 37°C, followed by two washes with PBS. Cells were plated at a high cell density (2 × 10^6/ml) in GM and allowed to attach for 5 h, washed with PBS twice, and switched to SFM for 16–24 h. The cultures were then fixed and stained with PI as described below and scored for NAN by fluorescence microscopy, as described above.

Analysis of the DNA Fragmentation. Cells were plated in 60-mm dishes (Costar), incubated in GM until confluent and then switched to

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**Fig. 7.** Bcl-2 and Bax proteins in the mouse mammary gland. Top, Bcl-2 expression; bottom, Bax expression. Rest., nonpregnant; Preg., pregnant; Lact., lactating; Invol., involuting. The time of collection of the glands is indicated on the top, in days. The dashes indicate the position of the Bcl-2 and Bax proteins.

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F. Basolo and L. Fiore (Institute of Pathology, Pisa University, Pisa, Italy), personal communication.
Apoptosis


206 Apoptosis of Mammary Cells


