Extracellular Matrix Regulates Apoptosis in Mammary Epithelium through a Control on Insulin Signaling

Nadia Farrelly, Yi-Ju Lee, Janine Oliver, Caroline Dive, and Charles H. Streuli
School of Biological Sciences, University of Manchester, Manchester M13 9PT, United Kingdom

Abstract. Adherent epithelial cells require interactions with the extracellular matrix for their survival, though the mechanism is ill-defined. In long term cultures of primary mammary epithelial cells, a laminin-rich basement membrane (BM) but not collagen I suppresses apoptosis, indicating that adhesion survival signals are specific in their response (Pullan et al. 1996. J. Cell Sci. 109:631–642). We now demonstrate that the signal from BM is mediated by integrins and requires both the α6 and β1 subunits. In addition, a hormonal signal from insulin or insulin-like growth factors, but not hydrocortisone or prolactin, is necessary to suppress mammary cell apoptosis, indicating that BM and soluble factors cooperate in survival signaling. Insulin induced autophosphorylation of its receptor whether mammary cells were cultured on collagen I or BM substrata. However, both the tyrosine phosphorylation of insulin receptor substrate-1 and its association with phosphatidylinositol 3-kinase were enhanced in cells cultured on BM, as was the phosphorylation of the phosphatidylinositol 3-kinase effector, protein kinase B. These results suggest a novel extracellular matrix–dependent restriction point in insulin signaling in mammary epithelial cells. The proximal signal transduction event of insulin receptor phosphorylation is not dependent on extracellular matrix, but the activation of downstream effectors requires adhesion to BM. Since phosphatidylinositol 3-kinase was required for mammary epithelial cell survival, we propose that a possible mechanism for BM-mediated suppression of apoptosis is through its facilitative effects on insulin signaling.

Key words: extracellular matrix • mammary gland • apoptosis • integrin • insulin

Cells in multicellular organisms monitor their environment through interactions with neighboring cells and through receptors for both soluble and locally acting factors including extracellular matrix (ECM). In addition to providing signals for proliferation, differentiation, and migration, such interactions are also essential for survival and in their absence, cells undergo apoptosis. While the cellular signaling pathways that control the decision to enter apoptosis are currently being studied extensively, little is known about the interplay between survival factors derived from the soluble milieu and the ECM.

The mammary gland is a particularly useful system to study mechanisms that regulate apoptosis. Most of its epithelial cells are removed by apoptosis during involution of the gland after lactation, and the survival of purified mammary epithelial cells can be manipulated in primary culture (Strange et al., 1992; Pullan et al., 1996). Several models have recently emerged to explain the post-lactational induction of apoptosis. One possibility is that cessation of suckling results in hormonal changes in the nursing mother that alter the supply of soluble cell survival factors. An example of this, withdrawal of soluble survival factors such as insulin or insulin-like growth factor I (IGF-I) from cultured cells results in apoptosis (Rodriguez-Tarduchy et al., 1992; Harrington et al., 1994; Parrizas et al., 1997). In the mammary gland in vivo, the expression of an IGF-binding protein which inhibits IGF action, IGFBP-5, is dramatically increased after weaning and may therefore act to trigger apoptosis (Tonner et al., 1997). Alternatively, expression of matrix-degrading metalloproteinases after weaning may trigger cell death by breaking down cell–ECM interactions (Talhouk et al., 1992; Alexander et al., 1996; Lund et al., 1996). Although these hypotheses offer plausible explanations for the induction of apoptosis in vivo, none have been proven, suggesting that the mechanism may be more easily deciphered using a cell culture model. However, even in cell cultures, very little is currently known about the control of mammary epithelial apoptosis.
Epithelial cells undergo apoptosis if they are denied contact with ECM (Frisch and Francis, 1994; Khwaja et al., 1997) and indeed, ECM is a survival factor in a number of cell types (Frisch and Ruoslahti, 1997). The ECM is linked to the cytoskeleton and to cytoplasmic signaling pathways through integrins, and both integrins and some of their downstream effectors, including p125FAK, are involved in the survival response (Frisch et al., 1996). However, there are very few natural situations where simple epithelial cells are denied contact with the ECM for appreciable periods of time, and we have therefore developed a more physiological model than previously used to examine ECM survival signaling. Primary cultures of mouse mammary epithelial cells and mammary cell lines show a differential long term apoptotic response to different ECM in tissue culture (Boudreau et al., 1995; Pullan et al., 1996). On collagen I-coated substrata these cells form organized cobblestone monolayers, express E-cadherin and desmocalins at their cell-cell junctions, and are polarized (Streuli, 1995; Unswicke et al., 1996). However, when cultured on specialized ECM known as basement membrane (BM), which contains laminin, collagen IV, nidogen and perlecan, they develop into three-dimensional structures which mimic alveoli in vivo (Aggeler et al., 1991). Although in both situations they interact with the respective substrata through integrins, cells cultured on collagen I show extensive apoptosis over periods of several days whereas those in contact with BM do not (Pullan et al., 1996). Since mammary cells interact with collagen and BM through different integrin receptors, it is likely that only certain integrins deliver a survival signal (Howlett et al., 1996). Since mammary cells interact with collagen I, collagen II and fibrinogen through different integrin receptors, it is likely that only certain integrins deliver a survival signal (Howlett et al., 1996).

Recent models for integrin-mediated control of phenotypic in adherent cells have indicated that integrins might cooperate with soluble factors to drive signaling pathways, possibly through the formation of multi-protein adhesion complexes (Miyamoto et al., 1996; Schneller et al., 1997). Indeed, both proliferation and differentiation (Streuli et al., 1995; Zhu et al., 1996) are dependent on ECM as well as growth and differentiation factors. For example, cell anchorage regulates the activation of mitogen activated protein kinase by serum or EGF, and epithelial cell interactions with BM are necessary for prolactin to trigger the kinase Jαk2 and activate the transcription factor Stat5 (L in et al., 1997; R enshaw et al., 1997; E dwards et al., 1998).

In this paper we have sought to determine whether soluble factors and BM act coordinately to regulate the suppression of apoptosis in primary cultures of mammary epithelium. We show that insulin is an essential survival ligand but that the cell–ECM interactions that lead to survival are necessary for insulin to trigger its downstream survival signaling pathway.

Materials and Methods

Cell Culture

Primary mammary epithelial cells were prepared from 14.5–18.5-d pregnant ICR mice as previously described (Pullan et al., 1996). After culture for 3 days, cells were trypsinized, passed through a 70-μm nylon mesh and washed extensively before usage or in some experiments commercially precoated Biocoat dishes were used (Becton Dickinson). A factor-reduced preparation of the BM matrix was prepared using sequential ammonium sulfate precipitations (Taub et al., 1990) and coated onto tissue culture plastic at 14 mg/ml. In some experiments, factor-reduced BM matrix was diluted into the culture medium (final concentration 100 or 400 μg/ml) and overlaid onto first passage cells plated on collagen I (Streuli et al., 1995).

Apoptosis Assays

To analyze DNA integrity, first passage cells were cultured in medium containing appropriate hormones and inhibitors for two days, then washed over a period of 2 h to remove the accumulated dead cells and debris. Fresh medium was added to cells and after 4 h newly apoptotic cells were collected from the medium and pooled with any remaining attached cells. DNA was extracted from samples (Pullan et al., 1996), its OD was measured at 260 nm, and separated on a 1% agarose gel to confirm DNA loading after staining the gel with ethidium bromide. Equal amounts of DNA were separated by conventional agarose gel electrophoresis, southern blotted, andapolitotic DNA. Ladders were visualized by hybridization with a digoxigenin-labeled total mouse genomic DNA probe (Boehringer Mannheim).

Single cell cultures were prepared by staining first passage cells through a 20-μm mesh, pelleting and resuspending in factor-reduced BM matrix at 2–4 × 10^6 cells/ml, as described (Streuli et al., 1991). 100 μl of the mammary cell suspension was gelled for 60 min on dishes precoated with a thin layer of BM, then cultured in serum-free D M E M / F 12 medium containing appropriate hormones, inhibitors or antibodies for 2 d before fixation in 2% paraformaldehyde. 25-μm cryosections were stained with 0.5 μg/ml Hoechst 33258 and apoptotic nuclei were counted in the single cell population. Each experiment was repeated two to three times and in each experimental condition, >600 single cells were scored for apoptosis. Polyclonal rabbit anti-αβ integrin antibody was prepared in this laboratory (E dwards and Streuli, 1998). It was shown to inhibit the adhesion of first passage mouse mammary epithelial cells to laminin, collagen IV, but not fibronectin or vitronectin. Purified rat monoclonal antibody to α6 integrin (G oH3) and its rat IgG2a isotype control were obtained from Sero tec. Rabbit polyclonal anti-laminin antibody was raised against purified laminin isolated from E H S tumor (a kind gift of P. Yurchenco, Robert Wood Johnson Medical School, Piscataway, NJ). This laminin had previously been shown to contain laminin A and B chains but not collagen IV or nidogen (Streuli et al., 1995). A protein A-derived IgG fraction from the rabbit serum was subsequently purified on an E H S affinity column. This antibody significantly inhibited the adhesion of first passage mouse mammary epithelial cells to laminin at 5 μg/ml and completely inhibited adhesion at 30 μg/ml (data not shown).

A quantitative assay for apoptosis based on measuring cellular detachment from different substrata was previously described (Pullan et al., 1996). Cells detaching into the medium of first passage cultures over periods of 4 h were pelleted and counted using either a hemocytometer or a Coulter counter. Detached cells were confirmed to have apoptotic morphology by fluorescence microscopy after staining with 0.5 μg/ml Hoechst 33258 and the remaining monolayers were also stained with Hoechst 33258 to confirm that apoptotic cells did not accumulate there. Quantitative data for detachment assays are all relative to the number of cells that attached to each substrata after plating.
Survival of Mammary Epithelium Is Regulated Coordinate by Insulin and BM

Mammary epithelial cells require appropriate cell–ECM interactions for survival since they undergo apoptosis in the absence of a BM (Pullan et al., 1996). To determine the mechanism by which BM suppresses apoptosis, we asked whether BM was able to act as a survival ligand by itself or whether additional factors were required to prevent cell death. First passage mammary epithelial cells isolated from midpregnant mice were plated on substrata of collagen I or a reconstituted BM matrix derived from the EHS tumor. Culture on a BM matrix in the presence of lactogenic hormones, insulin, prolactin, and hydrocortisone, suppressed apoptosis, whereas cells died on collagen I (Fig. 1A). However, removal of lactogenic hormones from the medium resulted in apoptotic death of the cells, even those cultured on the BM matrix (Fig. 1A). This indicated that survival of mammary cells required two signals, one from the BM and a second signal from the hormonal milieu.

To determine which of the three hormones acted in a survival capacity, cells plated on BM were incubated with combinations of insulin, prolactin, and hydrocortisone and the extent of cell death was measured by DNA fragmentation. A apoptosis occurred only when the cells were cultured without insulin (Fig. 1B). We also used an alternate assay for apoptosis, where single mammary cells were cultured within a BM gel, but apart from each other so that they were unable to form cell–cell interactions (Streuli et al., 1991). In this assay, apoptosis was measured by determining nuclear morphology after culture for 48 h (Fig. 1C).

Results

Analysis of DNA isolated from cells cultured on collagen I or BM in the presence of 1, 10, or 100 nM IGF-I or IGF-II. Lanes 1-3 additionally contained prolactin and hydrocortisone (p and h). Quantitative analysis of IGF-I suppression of apoptosis in single cells embedded within BM. Results are the mean ± SE of counts from six independent cultures from two separate experiments. The values with insulin and IGF-I are significantly different to those without hormones (*P < 0.05). Less than 5% of the cells were apoptotic at the time of plating (T0).

SDS-PAGE on 6% gels, and the gels were stained with Coomassie Brilliant Blue to normalize protein levels. Equal amounts of protein were immunoprecipitated either with anti–IRS-1 antibody (UBI) or anti-insulin receptor β antibody (Transduction Laboratories) and 20 µl protein A-Sepharose beads (Zymed Laboratories), before being separated on 6% gels under reducing conditions and transferred to Immobilon-P membranes (Millipore). Phosphotyrosine blots were blocked in 1% BSA in TBST (50 mM Tris-Cl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) and incubated overnight with monoclonal antibody 4G10 (UBI) at 1:1,000 for insulin receptor β or 1:3,000 for IRS-1. Interaction of the p85 subunit of PI 3-kinase with IRS-1 was examined by probing IRS-1 immunoblots with anti–PI 3-kinase (UBI). For each experiment, equal amounts of protein were confirmed for IRS-1 by blocking IRS-1 immunoblots in 3% nonfat dry milk/PBS and probing with 1:3,000 anti–IRS-1 antibody; for insulin receptor β, whole cell lysate blots were blocked with 3% BSA/TBST and probed with 1:250 anti-insulin receptor β. Protein kinase B (PKB; otherwise known as c-Akt) was analyzed by immunoblotting equal amounts of cell protein with either an anti–PKB antibody or an antibody specific for phosphorylated PKB (both at 1:2,000; New England Biolabs). Proteins were visualized by enhanced chemiluminescence (Amersham).

Mammary epithelial cells were plated on substrata of collagen I (Ci) or BM in the presence (+) or absence (−) of insulin, prolactin and hydrocortisone (i, p, and h). Note that DNA fragmentation is only suppressed in cells cultured on BM together with lactogenic hormones. (B) Southern analysis of DNA isolated from cells cultured on BM with combinations of insulin, prolactin and hydrocortisone. Samples in lanes 1-3 were from cells cultured in phenol red-free media, and the results indicate that estrogenic activity of phenol red did not contribute to survival. (C) Cryosections of cells suspended within gels of BM matrix after Hoechst staining (upper panels) and the corresponding differential interference contrast images (lower panels). These images are typical of apoptotic single cells cultured with hydrocortisone and prolactin (p and h), and those which resisted apoptosis when cultured additionally with insulin (i, p, and h). (D) Quantitative analysis of apoptosis in single cells embedded within BM. The percentage of apoptotic cells, as determined by nuclear morphology after Hoechst staining, is shown in cultures incubated with combinations of insulin, prolactin and hydrocortisone. Results are the mean ± SE of counts from five independent cultures from two separate experiments. The values with insulin are significantly different to those without insulin (*P < 0.05). Less than 5% of the cells were apoptotic in embedded cultures that were fixed 1 h after plating (T1). (E) Southern analysis of DNA isolated from cells cultured on collagen I or BM in the presence of insulin are significantly different to those without insulin (*P < 0.05). Less than 5% of the cells were apoptotic at the time of plating (T0).

**Results**

Survival of Mammary Epithelium Is Regulated Coordinate by Insulin and BM
20–30% of single cells were apoptotic providing insulin was present in the medium. However, in the absence of insulin, cell death increased significantly (P < 0.01) to 60–70% (Fig. 1 D). These two different approaches collectively demonstrate that BM does not act alone to suppress apoptosis in primary cultures of mammary epithelia. Instead, it regulates survival in combination with signals elicited by insulin. Furthermore, the data show survival is not dependent on mammary differentiation, since removal of prolactin and hydrocortisone, which are required for milk protein gene expression, did not result in apoptosis.

In addition to the ability of insulin to interact with its own receptor, this hormone can also bind to the IGF receptor although with a 100–1,000-fold lower affinity (Parrizas et al., 1997). IGF-I or IGF-II have been shown to act as survival factors in other cell types (Rodriguez-Tarduchy et al., 1992; Harrington et al., 1994; Kulik et al., 1997), and we therefore tested whether they could directly suppress mammary apoptosis. A physiological concentration of IGF-I and IGF-II significantly inhibited the DNA fragmentation exhibited by cells cultured on a BM matrix in the absence of other hormones (Fig. 1 E). Moreover, IGF-I suppressed apoptosis (P < 0.01) in single mammary cells cultured within the BM gel to the same extent as insulin (Fig. 1 F). The survival of mammary epithelia can therefore be regulated by signals from IGF-I and IGF-II in addition to those from insulin, and these signals act coordinately with BM.

**Laminin Is a Survival Ligand for Mammary Epithelial Cells**

Mammary epithelial cells plated onto a BM substratum form multicellular, alveolar-like structures (Barcellos-Hoff et al., 1989; A ggeler et al., 1991). Thus, it was important to ask whether this complex three-dimensional structure was involved with mammary cell survival, or if signals from the BM–integrin interactions were sufficient. Therefore, we cultured mammary cells as monolayers on collagen I and incubated them with the EHS BM preparation diluted into the culture medium. Under these conditions, the cells did not form alveoli but remained as monolayers on the culture dish with some matrix proteins precipitating over the cells (Streuli et al., 1995). Dilution of the BM preparation 140-fold to 0.1 mg/ml resulted in significant suppression of apoptosis, both in a quantitative assay (Fig. 2 A) and in DNA fragmentation studies (Fig. 2 B). BM proteins diluted 35-fold to 0.4 mg/ml suppressed apoptosis to virtually the same extent as in the cells cultured as alveoli on top of a BM substratum (Fig. 2 A and B). Thus, the three-dimensional multicellular structure is not a primary determinant of mammary cell survival.

BM contains several ECM proteins and one component, laminin, has previously been shown to be a survival ligand for other cell types (Kim et al., 1994; Vachon et al., 1996; Bozzo et al., 1997). To determine whether laminin was a survival factor for mammary epithelial cells we developed a function-blocking anti-laminin antibody that inhibited adhesion of mammary cells to laminin (data not shown). We assessed whether this antibody was able to interfere with the survival of mammary cells that had been cultured as single cells within BM gels (Fig. 3 A). We found that when the anti-laminin antibody was included in the gel, mammary cells underwent apoptosis even when they were cultured in the presence of insulin. In contrast, an adhesion-blocking antibody directed against fibronectin had no effect (Fig. 3 A), neither did the inclusion of an RGD peptide in the assay (Fig. 3 B). These results demonstrated that within the context of a three-dimensional BM gel, laminin has a survival-promoting function. Indeed, they show that both insulin and laminin can act as survival ligands for mammary epithelial cells, although they do not rule out the possibility that other soluble factors or ECM proteins may have a similar function.

**BM Regulates Mammary Survival through α6 and β1 Integrin Subunits**

Major cellular receptors for BM proteins, including laminin, are the transmembrane heterodimeric integrin receptors. To confirm that BM acts directly to suppress apoptosis, and that the survival signals were mediated by integrins, we assessed whether function-blocking anti-integrin antibodies interfered with the mammary cell survival using the single cell assay. In experiments where an adhesion-blocking polyclonal anti–mouse β1 integrin antibody was included in the gel, insulin failed to suppress apoptosis. Indeed, the levels of death were similar to those observed in single mammary cells in the absence of insulin (Fig. 4 A). One receptor for laminin is α6β1 integrin, and we therefore performed parallel experiments using a function-blocking anti–α6 integrin antibody (Fig. 4 B). The cellular
response was similar to that for the anti-β1 integrin antibody, providing evidence that both the α6 and β1 integrin subunits can transduce survival signals in normal mammary epithelial cells. Suitable antibodies to other mouse integrin subunits were not available to test for specificity. Therefore, we performed similar experiments using function-blocking anti-human integrin antibodies and demonstrated that α6 and β1 integrin, but not α2 integrin, acts as survival receptors in primary cultures of human mammary cells (Oliver, J., M. O’Hare, and C.H. Streuli, manuscript in preparation). In addition, our results with anti-fibronectin antibodies and RGD peptides do not support a role for αv integrins in mammary cell survival.

Our combined data using function-blocking antibodies to laminin and to integrin subunits show that suppression of apoptosis in mammary epithelia depends on interactions of the cells with BM through the α6 and/or β1 integrin receptors, and that laminin can act as a survival ligand.

**BM and Insulin Regulate Survival in Mammary Cells by Cross Talk of Signaling Pathways**

Since both insulin and laminin had been identified as mammary cell survival factors, it is possible that they trigger separate pathways required for suppressing apoptosis. However, recent work on the control of cell cycle and differentiation indicates that ECM affects the ability of growth factors/cytokines to trigger their downstream signaling kinases (Lin et al., 1997; Renshaw et al., 1997; Edwards et al., 1998). Thus an alternative mechanism to explain survival signaling in mammary cells is that the insulin- and BM-triggered pathways converge. To determine whether this was indeed the case, the extent of tyrosine phosphorylation in the proximal components of the insulin signaling pathway was measured (Fig. 5).

Insulin interacts with its receptor, causing receptor oligomerization and activation of the kinase domain. This results in tyrosine phosphorylation of the receptor β subunit. The adaptor protein insulin receptor substrate-1 (IRS-1) is recruited to the insulin receptor, then becomes tyrosine phosphorylated creating SH2 domain interaction sites for downstream enzymes such as PI 3-kinase (Myers et al., 1994; Sun et al., 1992). The ability of insulin to signal in primary cultures of mammary cells plated on BM was compared with those on collagen I. Tyrosine phosphorylation of the insulin receptor β subunit occurred within 15 min of insulin treatment and was independent of cell–ECM interactions (Fig. 5A). However, the ability of IRS-1 to become tyrosine phosphorylated in response to insulin was strongly dependent on cell interactions with BM, and virtually no IRS-1 phosphorylation was observed in cells cultured on collagen I (Fig. 5B).

It has been shown in several cell types that PI 3-kinase is required for preventing apoptosis, and that in neuronal cells and fibroblasts IGF-I suppresses apoptosis through

![Figure 3](image-url) Laminin affects the survival potential of mammary epithelial cells. (A) Quantitative analysis of apoptosis in single cells embedded within BM in the presence of anti-laminin and fibronectin antibodies. The percentage of apoptotic cells, as determined by nuclear morphology after Hoechst staining, is shown. Results are the mean ± SE of counts from four independent cultures from two separate experiments. The values with anti-laminin antibody are significantly different to those for control IgG and anti-fibronectin antibody (P < 0.001). Note that insulin together with prolactin and hydrocortisone failed to suppress apoptosis in mammary cells cultured with anti-laminin antibody. (B) Quantitative analysis of apoptosis in single cells embedded within BM in the presence of GRGDS and its control GRGES peptides. Note that there was no significant different difference in levels of apoptosis between those cells cultured with and without GRGDS (P = 0.34).

![Figure 4](image-url) Requirement of integrin for mammary cell survival. Quantitative analysis of apoptosis in single cells embedded within BM in the presence of anti-integrin antibodies. The percentage of apoptotic cells, as determined by nuclear morphology after Hoechst staining, is shown. Results are the mean ± SE of counts from five independent cultures from two separate experiments. The values with anti-integrin antibody are significantly different to those for control IgG (*P < 0.05; **P < 0.01). Insulin failed to suppress apoptosis in mammary cells cultured with either (A) anti-β1 integrin or (B) anti-α6 integrin antibody.
Quantitation of the data indicated that threefold more PI 3-kinase bound IRS-1, but the extent of the interaction of its p85 subunit with IRS-1 (Myers et al., 1994). PI 3-kinase did not associate with IRS-1 in mammary cells cultured on collagen I (Fig. 5 C). However, within 15 min of insulin treatment PI 3-kinase bound IRS-1, but the extent of the interaction was strongly dependent on cell adhesion to BM; quantitation of the data indicated that threefold more PI 3-kinase bound to IRS-1 in cells cultured on a BM substrate than in cells cultured on collagen I (Fig. 5 D).

One downstream target of PI 3-kinase signaling pathway is PKB, which has previously been implicated in the suppression of apoptosis (Dudek et al., 1997; Kuffermann-Zeh et al., 1997; Kulik et al., 1997; Crowder and Freeman, 1998). This enzyme is recruited to the plasma membrane following PI 3-kinase phosphorylation of membrane lipids, and then activated phosphorylation on serine/threonine residues (Downward, 1998). Using an antibody specific for phosphorylated PKB, we found that maximal activation only occurred when mammary cells treated with insulin had been cultured on BM (Fig. 6). Wortmannin completely abrogated insulin-induced PKB phosphorylation. Since many of the assays to measure mammary apoptosis in our work were carried out over several days, we also measured PKB phosphorylation status in cells cultured with insulin for 3 days. PKB was only phosphorylated in cells cultured on BM, even though its level remained constant whether the cells were on collagen I or BM substrata. These results are an indirect confirmation that PI 3-kinase activity depends on coordinate signals from insulin and ECM. Most importantly, they demonstrate that the PKB pathway, already shown to be a critical determinant of survival in other cell systems, relies on converging insulin and ECM signals in mammary epithelia.

Thus, the ECM dependence of insulin signaling correlates with the ability of BM, but not collagen I, to deliver survival cues.

**Pharmacological Inhibitors of Enzymes in the PI 3-Kinase Signaling Pathway Induce Apoptosis of Mammary Epithelial Cells**

The experiments described above suggested that the mechanism through which cell–ECM interactions regulate apoptotic fate in mammary epithelial cells is through a control on insulin-mediated PI 3-kinase signaling. To determine whether the PI 3-kinase pathway, or related pathways, were required for survival, the effects of the pharmacological inhibitors LY 294002 and wortmannin were examined in two independent assays for mammary apoptosis.

Mammary cells were cultured on a BM matrix with 10–100 nM IGF-I, with or without 0.1 or 1 μM LY 294002. In the absence of soluble factors the cells underwent apoptosis (Fig. 7 A, lane 2), and this was suppressed by IGF-I (Fig. 7 A, lanes 3–6). However, IGF-I failed to suppress apoptosis when LY 294002 was included in the cultures (Fig. 7 A, lanes 7–10). We observed low levels of apoptosis at 0.1 μM and very extensive DNA fragmentation at 1 μM LY 294002, a concentration previously shown to inhibit IGF-I signaling and PI 3-kinase activity (Vlahos et al., 1994; Parrizas et al., 1997). Similar results were obtained with wortmannin (data not shown).

The effect of the kinase inhibitors on suppression of apoptosis by insulin was also examined in the single cell assay. As before, mammary cells cultured within BM matrix were not able to survive in the absence of insulin. Insulin rescued the cells from apoptosis, but low levels of wortmannin (1 μM) and LY 294002 (0.1 μM) blocked survival signaling (Fig. 7 B).
is fairly broad but includes PI 3-kinase whereas LY 294002 is specific for PI 3-kinase (Vlahos et al., 1994; Ui et al., 1995). Thus, these two experiments indicate that the PI 3-kinase class of enzymes are necessary for mammary cell survival.

Together our results suggest that mammary epithelial cell survival depends upon a functional PI 3-kinase signaling pathway which is normally triggered by insulin when the cells are cultured with a BM substratum. Restricting the activity of this pathway by culture on collagen I results in cell death.

**Discussion**

The mechanisms that regulate death and survival decisions in epithelial cells are not understood. Yet these are fundamental cellular processes and are essential not only for deciphering the control of normal development, but also for designing strategies to tackle progressive diseases such as breast cancer.

In this study, we have addressed the mechanism whereby BM suppresses apoptosis in primary cultures of mammary epithelial cells and found that this type of ECM controls the ability of insulin to deliver survival signals. We have demonstrated that insulin is necessary to prevent death of mammary cells through the PI 3-kinase pathway, and furthermore that insulin-mediated interaction of PI 3-kinase with IRS-1 and phosphorylation of PKB, a key enzyme downstream in the PI 3-kinase pathway, is influenced by cell interactions with BM. This provides new evidence for the importance of ECM in the growth factor-mediated regulation of cell phenotype. Since α6 and β1 integrins are necessary for mammary cell survival, our work supports the notion that one function of integrins is to regulate growth factor signaling.
Two Signals Are Required for Survival of Mammary Epithelial Cells

Our previous studies, and those of others, have demonstrated that mammary epithelial cells depend on BM for survival (Boudreau et al., 1995; Pullan et al., 1996). We have now found that they require a further signal from soluble ligands to remain alive. Of three hormones tested, insulin, but not hydrocortisone or prolactin, was found to be necessary for suppression of apoptosis (Fig. 1). This survival effect of insulin could be mimicked by IGFs.

Insulin and IGFs have been shown to rescue apoptosis in several cell lines following interleukin-3 or serum withdrawal (Rodriguez-Tarduchy et al., 1992; Harrington et al., 1994; Parrizas et al., 1997). However, although IGFs have been proposed to act as survival factors in mammary gland, this has not been demonstrated directly. For example, overexpression of des(1-3)hIGF-I or IGF-I in mammary glands of transgenic mice delayed involution and the glands showed lower levels of apoptosis, but the target cells were not identified (Hadsell et al., 1996; Neuen-schwander et al., 1996). In the experiments performed in our study, purified populations of mouse mammary epithelial cells survived in the presence of insulin or IGFs, indicating that the factors suppress apoptosis by a direct action on the epithelial cells themselves.

Glucocorticoids have also been demonstrated to rescue cells from apoptosis in the mammary gland, although it was not determined whether the hormone acts directly on epithelial cells or through an indirect mechanism (Feng et al., 1995; Lund et al., 1996). In purified mammary epithelial cells hydrocortisone had a minimal effect on survival, and therefore its function in vivo would appear indirect. Thus, although in vivo studies have indicated a role for both IGFs and glucocorticoids in suppressing apoptosis in mammary gland, our study with isolated cells shows that only IGFs act directly on the epithelial cells themselves. IGFs are normally only synthesized by stromal cells in the mammary gland, but their expression is regulated by a synergy between growth hormone and estradiol (Yee et al., 1989; Ruan et al., 1995). Therefore, one explanation for the inhibition of apoptosis by hydrocortisone in vivo is that this steroid can act on stromal cells to alter IGF expression. Alternatively, since IGFBP-5 has been suggested to induce mammary involution, glucocorticoids may inhibit apoptosis in vivo by decreasing IGFBP-5 synthesis, as occurs in cultured fibroblasts (Conover et al., 1995; Tonner et al., 1997).

The ability of prolactin to suppress apoptosis directly in purified mammary epithelial cells was also tested. In agreement with previous studies which showed that prolactin could not rescue mammary apoptosis in vivo, we demonstrated that it also had a negligible effect on epithelial cell survival in culture (Feng et al., 1995; Marti et al., 1997). Prolactin regulates intracellular signaling through a Jak/Stat pathway, which is required for mammary differentiation. Although other cytokines have been proposed to suppress apoptosis through Stat transcription factors, it appears that this pathway is not directly involved in mammary cell survival (Fuji et al., 1997).

Together our data show that, of the hormones tested, insulin is sufficient for the survival of mammary cells cultured on BM. Since prolactin and hydrocortisone, in addition to insulin, are necessary for lactation, the results indicate that mammary cell survival does not depend on differentiation. Moreover, as cells on collagen I undergo apoptosis even in the presence of all three hormones, the survival mechanism would appear to have more to do with cell-ECM interactions than with differentiation.

Laminin Is a Survival Ligand for Mammary Cells

Cell-ECM interactions are known to suppress apoptosis in epithelial cells (Meredith et al., 1993; Frisch and Francis, 1994; Khwaja et al., 1997). However, such studies are based on comparing the survival potential of suspension-cultured epithelial cell lines with those adhering to ECM in short term assays. We have developed a model where primary mammary cells isolated directly from tissue are maintained on physiological substrata in longer term assays. These cells have not been selected for their ability to form cell lines and therefore retain an apoptotic potential that is as close as possible to that in vivo. They undergo apoptosis specifically after culture for several days on tissue culture plastic or collagen I, but not on BM (Pullan et al., 1996). The cells plated on a BM substratum form multilayered structures resembling alveoli in vivo (Barcellos-Hoff et al., 1989; A gelser et al., 1991). However, this three-dimensional architecture did not appear to play a role in suppression of apoptosis (Fig. 2). Under conditions where the cells remained as monolayers, apoptosis was efficiently suppressed by diluted BM proteins precipitating over the cells.

Further verification that BM had a direct survival signaling role came from studies using function-blocking antibodies. Using a single cell assay developed previously for analysis of differentiation (Streuli et al., 1991), we found that although insulin was able to significantly suppress apoptosis in cells cultured within a three-dimensional BM gel, it was not able to do so if anti-laminin antibodies were included (Fig. 3). Laminin has previously been shown to act as a survival ligand for other cell lineages including fibrosarcoma and neuroblastoma cells and has been implicated in preventing apoptosis during myogenesis (Kim et al., 1994; Vachon et al., 1996; Bozzo et al., 1997; Vachon et al., 1997). Our results extend these previous findings and show that laminin also has a survival function in mammary epithelium. However, laminin may not be a survival ligand for all cells, since endothelial cells underwent apoptosis on laminin substrate but not on fibronectin or vitronectin (Wary et al., 1996). Thus, distinct cell types have a different requirement for ECM to rescue them from apoptosis. It remains to be determined whether or not the survival response to different ECM ligands is mediated through similar signaling pathways.

Integrins Are Survival Receptors in Mammary Cells

In several cell systems, integrins have been shown to be required for mediating a survival response (Frisch and R oslatht, 1997). We tested the possibility that integrins delivered survival signals in mammary cells by examining the survival of single cells in BM gels after treatment with function-blocking antibodies, and found that both α6 and
integrins were necessary to prevent the cells from undergoing apoptosis. Although β1 integrins have been shown to be required for mammary epithelial cell survival, our results augment these studies by providing the first direct evidence that α6 integrin functions in such a pathway (Boudreau et al., 1995; Howlett et al., 1995; Fig. 4). These findings have recently been confirmed in our experiments with primary human breast epithelia, where anti–α6 and anti–β1 integrin antibodies induced cell death, although antibodies to the α2 subunit did not. The latter result indicates a specificity of response as mammary cells express antibodies to the cell survival. Furthermore, in preliminary immunostaining integrin but we found that it had no effects on mammary cell survival. In addition to α2 integrins we have only detected this subunit in the epithelial cells of virgin mammary gland and it appeared to be completely absent from pregnant and lactating tissue. Therefore, our data would not support the possibility that it is a survival receptor for mammary cells isolated from pregnant mammary gland, even though it does have this role in endothelia (Brooks et al., 1994; Scatena et al., 1998). In addition to the β1 integrin subunit, mammary cells also express β4 integrin, and both of these can partner the α6 subunit. Therefore, our data are not yet sufficient to confirm that laminin suppresses apoptosis through a direct cell interaction with the α6β1 integrin heterodimer, neither do they exclude the possibility that other integrin subunits are involved in survival. However, they do demonstrate that laminin is a ligand for survival and that the α6 and β1 subunits can act as survival receptors.

The metastatic breast cancer cell line, MDA-MB-435 expresses α6β1 integrin. Tumors induced by a derivative cell line in which α6β1 was functionally ablated were much smaller those of the parental cells, and showed a sixfold higher apoptotic/mitotic index (Wewer et al., 1997). These results suggest that α6 integrin provides an anti-apoptotic signal in vivo as well as in culture and together with the present study, they may explain why higher levels of α6 integrin in human breast carcinomas correlate with an increased likelihood of patient morbidity (Friedrichs et al., 1995). Moreover, they suggest that α6 integrin–ligand interactions might represent a target for therapeutic intervention in breast disease, especially in combination with agents which inhibit growth factor–mediated survival signals.

Receptor Cross Talk and the Control of Apoptosis

Since both BM and insulin were required for sustained prevention of apoptosis in mammary cells, it was possible that these ligands triggered either parallel or convergent survival pathways. A number of studies indicate that PI 3-kinase is essential for suppressing apoptosis in other cell systems, possibly through PKB which has been implicated as a downstream regulator of survival (Yao and Cooper, 1995; Minshall et al., 1996; Dudek et al., 1997; Kaffmann-Zeh et al., 1997; Kennedy et al., 1997; Khwaja et al., 1997; Kulik et al., 1997; Parrizas et al., 1997; Crowder and Freeman, 1998). Our experiments using inhibitors also demonstrated a requirement for PI 3-kinase in mammary cell survival (Fig. 7). Therefore, we examined whether this pathway was independently regulated by the two separate ligands or if BM controlled the ability of insulin to trigger its phosphorylation cascade, by measuring the levels of phosphotyrosine in its proximal signaling proteins.

Insulin rapidly induced tyrosine phosphorylation of its receptor. However, the signaling events downstream of the insulin receptor resulting in IRS-1 tyrosine phosphorylation and its association with PI 3-kinase were only propagated in cells cultured on BM and not on collagen I (Fig. 5). These results contrast with a study using CHO cells overexpressing insulin receptor, which showed that transient adhesion to fibronectin >20 min enhanced the insulin-induced phosphorylation of both the receptor and IRS-1 (Guilherme et al., 1998). Our data support the conclusion that ECM amplifies insulin signaling, but extends it by showing that once nontransfected primary epithelial cells have become established within an ECM environment over several days, they develop a selective sensitivity to growth factor signaling. Thus, the cellular environment determines whether the insulin signal can be propagated efficiently, and this only occurs when the cells contact a BM but not when they are on collagen I. Indeed, the results indicate the existence of an ECM-dependent restriction point in insulin signaling, which occurs downstream of insulin receptor phosphorylation.

The conclusion that BM and insulin cooperate to drive insulin signaling was confirmed in further experiments where we examined PKB phosphorylation, using both short and long term treatments with insulin (Fig. 6). Cell–matrix interactions had a similar effect of enhancing EGF signaling in MDCK cells, although in that study the PKB activation was examined in cells briefly attached to an ECM in comparison with those detached from a substrate (Khwaja et al., 1997). Together, these complementary results indicate that both the short and long term activation of enzymes in the PI 3-kinase signaling pathway requires adherent cells to be in the correct ECM microenvironment, which contributes to both EGF and insulin signaling.

At the current time we do not know whether PKB is absolutely required for mammary cell survival, but given the results from other cell systems, this seems likely. Candidates for the downstream link between PKB and the apoptotic machinery include glycercol synthase kinase-3, procaspase-9, and the pro-apoptotic protein Bad (Datta et al., 1997; del Peso et al., 1997; Cardone et al., 1998; Pap and Cooper, 1998). Although the phosphorylation of PKB and Bad have been dissociated from each other, primary cultures of mammary epithelial cells do express Bad, which therefore remains a potential target for regulation by PKB (Scheid and Uronio, 1998; our unpublished data).

A dhesion to ECM has previously been shown to suppress apoptosis in several cell types (Meredith et al., 1993; Frisch and Francis, 1994; Kim et al., 1994; Vachon et al., 1996; Wary et al., 1996; Bozzo et al., 1997; Khwaja et al., 1997; Farrellly et al., 1997; Minshall et al., 1996; Dudek et al., 1997; Kaffmann-Zeh et al., 1997; Kennedy et al., 1997; Khwaja et al., 1997; Kulik et al., 1997; Parrizas et al., 1997; Crowder and Freeman, 1998). Our experiments using inhibitors also demonstrated a requirement for PI 3-kinase in mammary cell survival (Fig. 7). Therefore, we examined whether this pathway was independently regulated by the two separate ligands or if BM controlled the ability of insulin to trigger its phosphorylation cascade, by measuring the levels of phosphotyrosine in its proximal signaling proteins.

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tors, or with integrin and downstream components in the
stratum for integrin and growth factor receptors, as has been demon-
strated for mouse mammary epithelial cells. This suggests that current
mice have demonstrated that apoptosis in the sin-
Al signals from soluble factors and ECM are also implicated in the
control of mammary differentiation, where prolactin and BM
are both necessary for triggering the prolactin signal-
ing cassette, Stat5 DNA binding, and milk protein gene
transcription (Streuli et al., 1995; E dwards et al., 1998).

A though our work shows that both laminin and insulin act as survival ligands for mammary cells, we cannot rule out the possibility that other types of ligand are important in regulating survival. Insulin, for example, was only able to suppress apoptosis of single mammary cells cultured within BM by about twofold, suggesting that other factors may play a role in mammary cell survival in vivo. Prelimi-
inary studies with primary cultures of human mammary ep-
ithelial cells, have demonstrated that apoptosis in the sin-
gle cell assay can be reduced by insulin, but is further suppressed by EGF. But with mouse mammary cells, EGF
does not have an additional survival effect over and above insulin (Oliver, J., and C.H. Streuli, unpublished data). Therefore, it may be that additional cytokines can sup-
press apoptosis still further than insulin and BM in the
mouse system. Even if other mammary survival factors re-
main to be identified, our data are still sufficient to demon-
strate that both insulin and integrin contribute to the survival of mammary cells.

In summary, these studies indicate that an important function of cell–ECM interactions is to modulate growth factor and cytokine responses. This suggests that current thinking about growth factor signaling in adherent cells should include a component from the ECM. One mecha-
nism might be via direct or indirect associations between integrin and growth factor receptors, as has been demon-
strated for αvβ3 integrin and the insulin and PDGF recep-
tors, or with integrin and downstream components in the
insulin signaling pathway, as shown with αvβ3 integrin and
IRS-1 (Vouri and Rouslahti, 1994; Schneller et al., 1997).
The role of integrins and the cytokoskeleton in signal trans-
duction may be to provide a scaffold whereby components of growth factors cassettes are assembled into discreet do-
 mains within the cell, so that they can propagate signals ef-
iciently. Indeed, a modular nature for membrane signal-
ing complexes has been proposed, which may explain the recruitment of signaling proteins to cytoskeletal structures such as adhesion plaques (Miyamoto et al., 1995; Plopper et al., 1995; Pa sson and Scott, 1997; Simons and Ikon en, 1997). Our current goal is to address this issue by deter-
mining whether the macromolecular organization of insu-
lin signaling proteins and integrins in mammary cells cul-
tured on BM are different to those in cells on collagen I.

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