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Requirement of basement membrane for the suppression of programmed cell death in mammary epithelium

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SUMMARY

Apoptosis is an active mechanism of cell death required for normal tissue homeostasis. Cells require survival signals to avoid the engagement of apoptosis. In the mammary gland, secretory epithelial cells are removed by apoptosis during involution. This cell loss coincides with matrix metalloproteinase activation and basement membrane degradation. In this paper we describe studies that confer a new role for basement membrane in the regulation of cell phenotype. We demonstrate that first passage epithelial cells isolated from pregnant mouse mammary gland die by apoptosis in culture, but that cell death is suppressed by basement membrane. The correct type of extracellular matrix was required, since only a basement membrane, not plastic or a collagen I matrix, lowered the rate of apoptosis. Attachment to a matrix per se was not sufficient for survival, since apoptotic cells were observed when still attached to a collagen I substratum. Experiments with individually isolated cells confirmed the requirement of basement membrane for survival, and demonstrated that survival is enhanced by cell-cell contact. A function-blocking anti-β_1 integrin antibody doubled the rate of apoptosis in single cells cultured with basement membrane, indicating that integrin-mediated signals contributed to survival. We examined the cell death-associated genes *bcl-2* and *bax* in mammary epithelia, and found that although the expression of Bcl-2 did not correlate with cell survival, increased levels of Bax were associated with apoptosis. We propose that basement membrane provides a survival stimulus for epithelial cells in vivo, and that loss of interaction with this type of matrix acts as a control point for cell deletions that occur at specific times during development, such as in mammary gland involution.

Key words: Mammary gland, Breast, Apoptosis, Bcl-2, Bax, Extracellular matrix, Basement membrane, Integrin

INTRODUCTION

The control of cell number and the removal of inappropriate or damaged cells is critical in developing and adult organisms. This regulation occurs via coordinated control of cellular proliferation, differentiation, and death. The mechanism of physiological and of much toxin- or drug-induced cell death in mammals is apoptosis (Dive and Wyllie, 1993; Thompson, 1995). It has been proposed that all cells require survival signals to avoid the engagement of apoptosis (Raff, 1992). Such survival signals include cell type-specific growth factors and cytokines, notably insulin-like growth factor-1, PDGF, nerve growth factor, and several interleukins (Collins and Rivas, 1993). In addition to soluble factors, cell-cell contact has been implicated as a determinant of cell survival (Bates et al., 1994), and the absence of extracellular matrix (ECM) has been shown to trigger apoptosis when cells are cultured on non-adhesive substrata (Meredith et al., 1993; Frisch and Francis, 1994).

In this study we demonstrate that for non-immortalised, early passage epithelial cells isolated directly from mammary gland, soluble trophic factors and cell-cell contact are not the only environmental controllers of cell survival and apoptosis, but that the basement membrane also contributes essential signals for survival.

The parenchyma of functional mammary gland contains three epithelial compartments: luminal epithelium, alveolar epithelium, and myoepithelium (Streuli, 1995). The luminal epithelium lines milk-collecting ducts and the cells are separated from basement membrane and from stromal ECM by a layer of myoepithelium. The alveolar epithelium, which is responsible for milk production, interacts directly with myoepithelial processes that form a sparse basket network around alveoli, and also with the laminin-rich basement membrane that separates both of these cell types from stroma. These cells undergo coordinated proliferation and death during normal mammary gland development. In the reproductive cycle, alveolar cells proliferate vigorously in pregnancy, differentiate at lactation, and die by apoptosis after weaning (Walker et al., 1989; Strange et al., 1992).

Hormones are the systemic regulators of mammary cell proliferation and lactation, but locally acting signals from the basement membrane are also required for the function of this tissue (Barcellos-Hoff et al., 1989; Schmidhauser et al., 1992; Streuli et al., 1991, 1995a,b). In addition to the role of basement membrane in milk protein production, changes in the organisation of the matrix may be involved in the onset of involution, where regression of the gland involves apoptotic cell loss. Following weaning, the epithelial basement membrane becomes increasingly folded and redundant (Walker et al., 1989), and activated metalloproteinases degrade the matrix associated with alveolar epithelium (Talhouk et al., 1992). The consequent loss of cell-matrix and cell-cell adhesions is associated with the imminent onset of apoptosis (Strange et al., 1992).

Since apoptotic cell death is a normal process in mammary gland development, we asked whether it was controlled directly by the extent and type of cell-matrix interactions. Our culture studies demonstrate that basement membrane provides a survival stimulus for first passage mammary epithelial cells, and that signals transduced by integrins are required for the suppression of apoptosis. In addition, we show that increased Bax expression is associated with cell death in mammary cells, indicating that in the absence of appropriate matrix-mediated survival signals Bax may contribute towards the execution of the apoptotic pathway.

MATERIALS AND METHODS

Cell culture

All experiments were performed with first passage mammary epithelial cells from 14.5-18.5 day pregnant ICR mice. Primary epithelial cultures were prepared from isolated mammary alveoli, and after 48-72 hours, cell monolayers were trypsinised to single cell suspensions, washed with serum-containing DMEM/F12 medium (Life Technologies Ltd, Paisley, UK), and plated at 1.0×10^5 to 2.25×10^5 cells per cm^2 in medium containing 5% fetal calf serum (Life Technologies), 5 μg/ml insulin (Sigma Chemical Co., Poole, UK), 10 ng/ml EGF (Promega Corporation, Southampton, UK), and 50 μg/ml gentamicin (Barcellos-Hoff et al., 1989; Streuli et al., 1991). The medium was subsequently changed to differentiation medium, which was DMEM/F12 containing the lactogenic hormones, 5 μg/ml insulin, 1 μg/ml hydrocortisone and 3 μg/ml ovine prolactin (Sigma), but neither serum nor EGF. M1 murine myeloid leukaemia cells were cultured in DMEM medium (Life Technologies) supplemented with 10% foetal calf serum.

Substrata

Collagen I-coated dishes were prepared by incubating plates overnight at 4°C with rat tail collagen in PBS to give a coating density of 8 $\mu g/cm^2$, washed twice with cold PBS and once with medium before use. EHS matrix was prepared from the Engelbreth-Holm-Swarm tumour and used as a substratum, as previously described (Streuli et al., 1991). In some experiments, EHS matrix was treated to remove the majority of growth factors by sequential precipitation with 20% ammonium sulphate, followed by dialysis (Vukicevic et al., 1992).

Time-lapse videomicroscopy

Time-lapse videomicroscopy was performed on cells cultured in flasks completely filled with differentiation medium, and maintained on a heated stage. The microscope optics were by Zeiss, and cells undergoing apoptosis were recorded at 1/160× real time.

Kinetics of cell detachment

At 24 hours after plating, the cultures were washed three times and incubated overnight in differentiation medium. On each of the following 3 days, cultures were washed to remove cell debris that accrued overnight, and the cells that subsequently detached were collected and counted every 4 hours. The nuclear morphology of these cells was routinely assessed (see below). These experiments were performed using 140 mm culture plates, and the data were adjusted to show the number of cells detaching in 4 hours per 1000 cells that initially adhered to the substratum. In some experiments, 10 ng/ml insulin-like growth factor-1 or 1 ng/ml TGF-β1 (R and D Systems, Abingdon, UK) were included in the media.

Nuclear morphology

Detached cells were stained with 0.5 μg/ml Hoechst 33258 (5 minutes, RT; Sigma) and examined by fluorescence microscopy. The nuclear morphology of cells remaining attached to the substratum was assessed by fluorescence microscopy after fixing cells in 1% formaldehyde and staining with Hoechst 33258 or 1 mg/ml 7-amino-actinomycin D (Molecular Probes Inc., Eugene, OR), both of which produced similar patterns of nuclear fluorescence in conventional microscopy. 7-Amino-actinomycin D was visualised in the confocal microscope (Bio-Rad MRC 600) by excitation at 514 nm using an argon ion laser. Photography was on Zeiss optics using T-Max 400 film.

Analysis of DNA integrity

DNA was extracted from cells at specific times during the detachment assay. Either cells that had detached from the substratum, or total cultures including detached cells and the remaining attached monolayers, were analysed. The DNA extraction protocol was adapted from that described by Tilly and Hseuh (1993). Pooled cell pellets were gently homogenised in 100 mM NaCl, 10 mM EDTA, 300 mM Tris-HCl, pH 8.0, 200 mM sucrose, and SDS was added to 0.65% before incubation for 30 minutes at 60°C. Proteinase K was added to 500 μg/ml and the sample incubated for 1 hour at 55°C. Potassium acetate was added to 500 μg/ml and the sample was placed on ice for 30 minutes. Following centrifugation at 4,500 rpm for 15 minutes (4°C), the supernatant was extracted once or twice with phenol:chloroform:isoamylalcohol (25:24:1, by vol.), then chloroform:isoamylalcohol (24:1, v/v), and the DNA was precipitated with cold absolute ethanol. The DNA was dissolved in 200 μl TE ( ), pH 8.0, and treated with 2.5 μg DNase-free RNase for 60 minutes at 37°C. Following further phenol extraction and ethanol precipitation, equal amounts of DNA were separated on 2% agarose gels before Southern blotting and hybridisation with ^{32}P- or digoxygenin-labelled mouse genomic DNA. Non-radioactive probe labelling and immunochemiluminescence detection were performed according to the manufacturer's instructions (Boehringer Mannheim UK, Sussex, UK).

RNA analysis

Total RNA was extracted from primary cultures of mammary epithelial cells using the guanidinium thiocyanate method, and northern analysis for the detection of specific mRNAs was performed as described (Streuli and Bissell, 1990). Hybond-N membranes were probed with ^{32}P-labelled cDNA fragments for *sgp-2* or *bax*, washed with 0.5× SSC, 0.2% SDS at 68°C, and exposed either to Kodak XAR film, or to Fujix Bas 2000 storage phosphorimaging plates for quantitative analysis. The *bax* cDNA fragment was obtained by reverse transcription of total RNA collected from adult mouse ovaries, followed by the polymerase chain reaction. The sequence of the forward primer used for amplification was 5′-GGTTTCATCCAGGATCGAGCAG-3′ (5′-appended *Eco*RI site), and the sequence of the reverse primer was 3′-ACAAAGATGGT-CACGGTCTGCC-5′ (5′-appended *Hin*dIII site). The reaction produced the expected cDNA fragment corresponding to bases 107-508 of the coding sequence, which was then subcloned into the pGEM4z vector (Promega).

Single cell apoptosis assay

Primary mammary cultures were trypsinised and single cells and cell clusters were suspended at 1×10^6 cells/ml within gels of collagen I or factor-free EHS matrix, as previously described (Streuli et al., 1991). After 2 days of culture in differentiation medium, the gels were fixed in PBS containing 2% paraformaldehyde, pH 7.4 (20 minutes, 37°C), washed in PBS containing 0.1M glycine (3 times, 20 minutes), then in PBS containing 0.1% Triton X-100, 0.05% Tween-20, 1% goat serum, and stained with 5 μg/ml Hoechst 33258 (1 hour, RT). The numbers of single cells, doublets, or clusters of more than 2 cells with normal or apoptotic nuclei were counted.

Function-blocking β_1 integrin assay

For these experiments a novel function-blocking anti-β_1 integrin antibody was used. The preparation of this antibody will be described elsewhere, but is summarised here in brief. $\alpha_4\beta_1$ integrin was affinity-purified under non-denaturing conditions from whole mouse embryos using Sepharose-conjugated PS/2, a rat monoclonal antibody specific for mouse α_4 integrin. The purified integrin was used to immunise rabbits, and IgG was isolated from the resulting sera. This antibody specifically recognised α_4 and β_1 integrins in western blotting. However, mammary epithelial cells do not express α_4 integrin (Delcommenne and Streuli, 1995) and the antibody therefore only recognised β_1 integrins and their associated α subunits in these cells. Preliminary adhesion assays with different ECM components indicated that the antibody blocked spreading and partially blocked attachment of mammary epithelial cells to laminin. To assess the role of β_1 integrins in adhesion-mediated survival signalling, first passage mammary epithelial cells were seeded in differentiation medium containing either 100 μg/ml or 200 μg/ml anti-integrin IgG or pre-immune rabbit IgG, or no antibody, onto coverslips coated with EHS matrix containing the same concentration of antibody. After 48 hours, the coverslips were washed and fixed in methanol:acetone (1:1, v/v) for 3 minutes at –20°C before air-drying, washing in PBS containing 0.1% Triton X-100, 0.05% Tween-20, 1% goat serum, and staining with 10 ng/ml Hoechst 33258 for 15 minutes.

Immunofluorescence

Mouse mammary tissue was frozen in liquid nitrogen and stored under liquid nitrogen or at –70°C until use. 5 μm cryosections were fixed with methanol:acetone (1:1, v/v), air-dried, rehydrated in PBS containing 1% goat serum, 0.2% Triton X-100, 0.05% Tween-20, and stained by indirect immunofluorescence with a 1/1,000 dilution of a rabbit antibody to a synthetic peptide (residues 41-54) of mouse Bcl-2 or with tissue culture supernatant of mouse monoclonal antibodies LL001 for cytokeratin 14, and LE41 for cytokeratin 8. Primary antibodies were detected with FITC-conjugated donkey anti-rabbit IgG or LRSC-conjugated donkey anti-mouse IgG (Jackson Immuno-Research Laboratories Inc, West Grove, PA). The specificity of the anti-Bcl-2 reaction was confirmed using excess peptide. Nuclei were counterstained with 0.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI).

Western blotting

Cells cultured on different substrata were treated with HBSS containing 2 mg/ml EDTA (5 minutes, 37°C), then with the same buffer containing 5 mg/ml trypsin (4-7 minutes, 37°C) (Sigma), washed with an equal volume of DMEM/F12 medium containing 10% serum (4°C), pelleted, washed in ice-cold PBS to remove serum, and snap frozen in liquid nitrogen. Cell pellets were lysed in 12.75 mM Tris-HCl, pH 6.8, 2% SDS, and samples were removed for protein estimation using the BCA assay (Pierce and Warriner, Chester, UK). Equal amounts of cell protein were separated by SDS-PAGE under reducing conditions. Gels were either stained with Coomassie Brilliant Blue to confirm equal loading of cell proteins, or transferred to Hybond-C extra (Amersham International plc, Little Chalfont, UK) or Immobilon-P (Millipore UK Ltd., Watford, UK). Membranes were treated with 2% skimmed milk (Marvel), 2% BSA, or 0.2% I-block (Tropix Inc., Bedford, MA), 1% polyvinylpyrrolidone (Sigma), 0.3% Tween-20 in PBS to block non-specific binding sites (overnight, 4°C) and incubated (2 hours, RT) with either 1/10,000 dilution mouse anti-rat β-casein antibody, 1/10,000 dilution of a rabbit antibody to Bcl-2, or 1.4 μg/ml of a rabbit antibody to Bax. The anti-Bax antibody was prepared in our laboratory by immunizing rabbits with a synthetic peptide of mouse Bax (residues 44-59), and IgG from the resulting serum was affinity purified on a Bax peptide column. In each case, the specificity of the anti-Bcl-2 and anti-Bax reactions were confirmed using excess peptide. Detection of casein and Bcl-2 were achieved with horseradish peroxidase-conjugated rat anti-mouse IgG or donkey anti-rabbit IgG (Sigma), followed by enhanced chemiluminescence (Amersham). Detection of Bax was achieved with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma), followed by chemiluminescence detection using CSPD (Tropix).

RESULTS

Mammary epithelial cells die by apoptosis in culture

During our initial attempts to clone luminal epithelial cells from pregnant mouse mammary gland, we noted that we were only able to obtain a very limited number of colonies and that it was not possible to subculture the cells. Examination of these first passage cells plated on collagen I or plastic and cultured with serum, EGF, and insulin, revealed significant cell death. Since mammary epithelial cells proliferate in the presence of serum and EGF, we subsequently studied the mechanism of cell death in the absence of proliferation, by replacing the growth medium with differentiation medium containing only the survival factor insulin and the lactogenic hormones hydrocortisone and prolactin. Cells that had initially formed confluent, organised monolayers did not contain proliferating cells when cultured in differentiation medium (see below), but deteriorated over a period of several days with many cells detaching from the monolayer (Fig. 1a,b,c). Time-lapse video microscopy indicated that these cells died by apoptosis and then detached: individual cells separated from their neighbours, and rapidly and asynchronously extended and withdrew cellular processes (Fig. 1d). Eventually their movements ceased, the cells shrank and they were released from their substratum in a manner characteristic of cells undergoing apoptosis (Evan et al., 1992). The detached cells exhibited typical apoptotic morphology with nuclei that were fragmented and condensed (Fig. 1e), and their DNA was cleaved into internucleosomal fragments (Fig. 1f), confirming that cell death was by apoptosis (Bortner et al., 1995).

These results show that epithelial cells from mammary gland can die by apoptosis in culture. Since this death occurred in confluent monolayers of cells that interact both with the substratum on to which they were plated and with each other, our data imply that adhesion neither to a matrix such as collagen I, nor to adjacent cells, is sufficient for survival.

Basement membrane suppresses apoptosis of mammary epithelium

In vivo, the alveolar epithelium interacts directly with a basement membrane. In culture, mammary epithelial cells form lactational alveolar structures when plated on an exogenous basement membrane (Barcellos-Hoff et al., 1989). To determine whether this type of matrix promotes cell

survival, we asked whether exogenous basement membrane could decrease the level of apoptosis in cultured mammary cells.

Mammary cells were plated with serum and EGF on collagen I, on plastic, or on a reconstituted basement membrane (EHS matrix). After 24 hours, the growth medium was replaced with differentiation medium, and on each of the following 3 days the cultures were washed and fresh medium was replaced every 4 hours. After each 4 hour period (chosen to prevent non-specific degradation of detached cells), total numbers of detached, apoptotic cells were counted. Cell detachment from collagen or plastic was up to 15 times faster than from basement membrane (Fig. 2a). Most cell detachment from the monolayer cultures occurred 1-2 days after addition of differentiation medium, and the lower level of detachment occurring in subsequent days was most likely due to the reduced number of cells remaining on the dish, since detachment (and proliferation) in the presence of serum remained

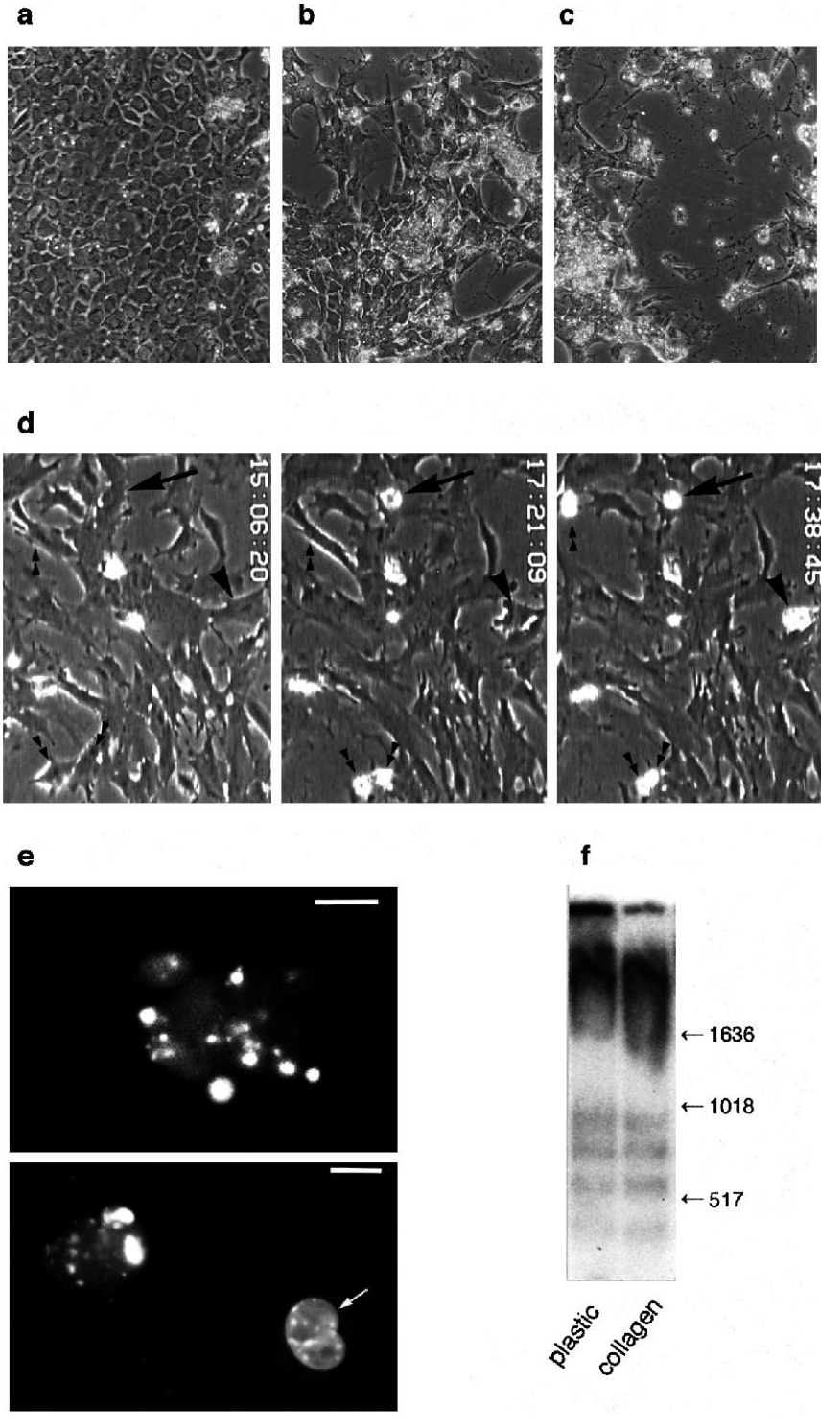

Fig. 1. Mammary epithelial cells died by apoptosis in culture. (a-c) Representative phase-contrast micrographs of mammary epithelial cells cultured on collagen I. (a) Cultures were confluent at the time of switch to differentiation medium. (b,c) They subsequently deteriorated as cells died and detached from the collagen substratum. The morphology of cells on a similar area of the same culture dish is shown after (b) 2 days, and (c) 4 days in differentiation medium. Bar, 100 μm. (d) Time-lapse videomicrographs of cells in differentiation medium prior to detachment from a plastic substratum. Arrows and arrowheads indicate individual attached and spread cells that rounded up, underwent a characteristic 'dance of death' (Evan et al., 1992), and eventually detached and moved into the culture medium. Rounding cells were never seen to undergo mitosis under these culture conditions. (e) Nuclear morphology of single detached cells collected from collagen I (upper panel) and plastic (lower panel) monolayer cultures, stained with Hoechst 33258. More than 80% of the detached cells showed the morphology of apoptosis with condensed and fragmented chromatin, while normal cells showing diffuse nuclear staining (arrow) were rarely seen, and there was no morphological evidence of mitosis. Bar, 10 μm. (f) DNA isolated from detached cells exhibited internucleosomal cleavage to 180 base-pair integers when separated by conventional agarose gel electrophoresis. Size markers in bp are shown in the margin. Data shown in a-f are representative of 2-6 repeat experiments.

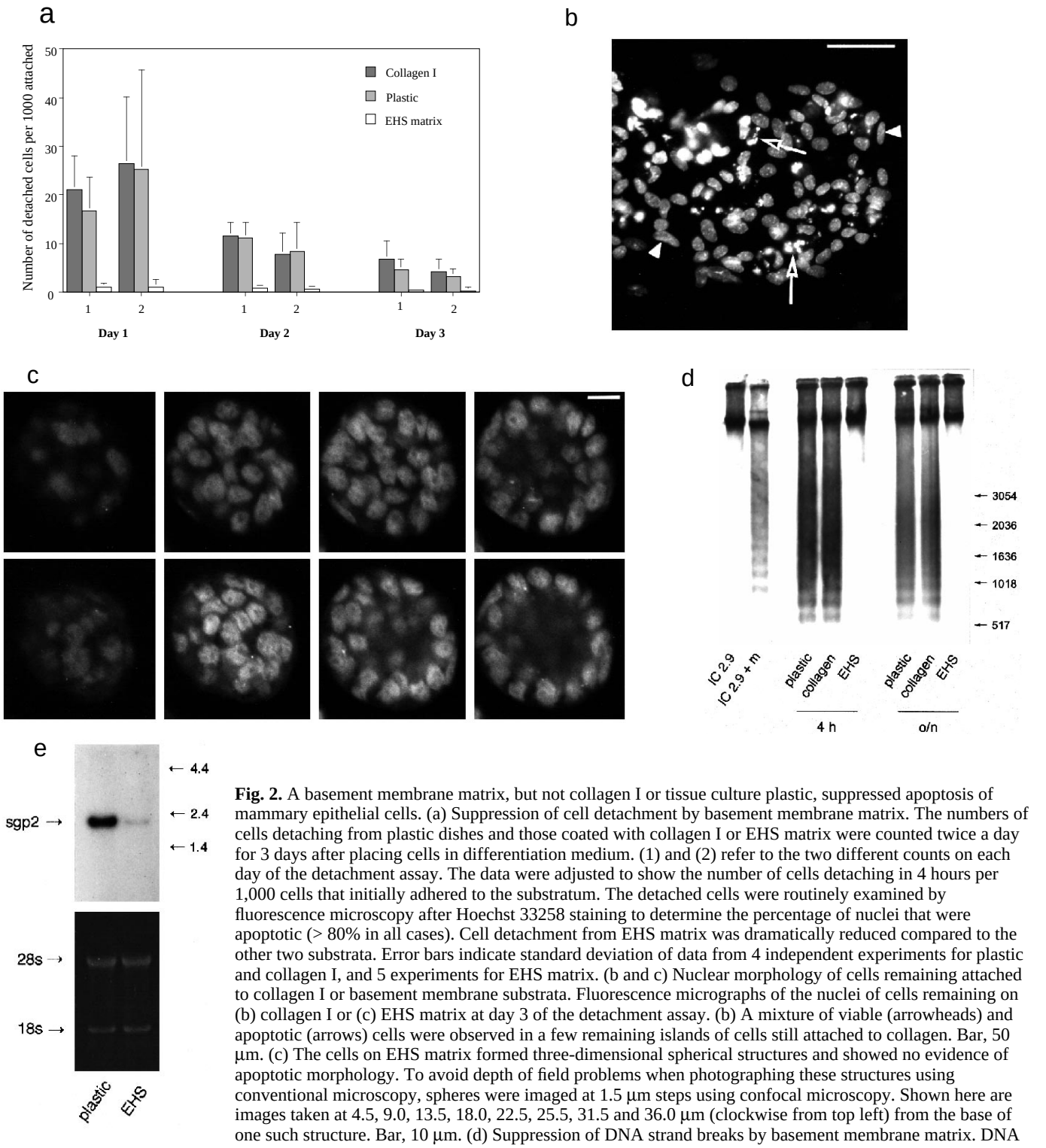

Fig. 2. A basement membrane matrix, but not collagen I or tissue culture plastic, suppressed apoptosis of mammary epithelial cells. (a) Suppression of cell detachment by basement membrane matrix. The numbers of cells detaching from plastic dishes and those coated with collagen I or EHS matrix were counted twice a day for 3 days after placing cells in differentiation medium. (1) and (2) refer to the two different counts on each day of the detachment assay. The data were adjusted to show the number of cells detaching in 4 hours per 1,000 cells that initially adhered to the substratum. The detached cells were routinely examined by fluorescence microscopy after Hoechst 33258 staining to determine the percentage of nuclei that were apoptotic (> 80% in all cases). Cell detachment from EHS matrix was dramatically reduced compared to the other two substrata. Error bars indicate standard deviation of data from 4 independent experiments for plastic and collagen I, and 5 experiments for EHS matrix. (b and c) Nuclear morphology of cells remaining attached to collagen I or basement membrane substrata. Fluorescence micrographs of the nuclei of cells remaining on (b) collagen I or (c) EHS matrix at day 3 of the detachment assay. (b) A mixture of viable (arrowheads) and apoptotic (arrows) cells were observed in a few remaining islands of cells still attached to collagen. Bar, 50 μm. (c) The cells on EHS matrix formed three-dimensional spherical structures and showed no evidence of apoptotic morphology. To avoid depth of field problems when photographing these structures using conventional microscopy, spheres were imaged at 1.5 μm steps using confocal microscopy. Shown here are images taken at 4.5, 9.0, 13.5, 18.0, 22.5, 25.5, 31.5 and 36.0 μm (clockwise from top left) from the base of one such structure. Bar, 10 μm. (d) Suppression of DNA strand breaks by basement membrane matrix. DNA (4 μg) isolated from cells cultured either for 4 hours (4 h) or overnight (o/n) in differentiation medium on plastic, collagen I or EHS matrix was separated by conventional agarose gel electrophoresis, Southern blotted, probed with a mouse genomic DNA-DIG labelled probe and immunochemiluminescently detected. Only the cells cultured on plastic and collagen I exhibited internucleosomal cleavage to 180 base-pair integers. The cells cultured on EHS matrix showed no evidence of DNA strand breaks, even after culture overnight. Untreated and melphelan-treated (m) IC 2.9 mouse mast cells are also shown as a positive control; these cells represent a well characterized model for the induction of apoptosis (Chapman et al., 1994). Size markers in bp are shown in the margin. (e) sgp-2 mRNA was upregulated in mammary epithelial cells on tissue culture plastic. Northern blot of 3 μg total RNA prepared from mammary cells cultured in differentiation medium for 1 day on tissue culture plastic and 2 days on EHS matrix. Equal loading was confirmed by ethidium bromide staining (lower panel). Blots were hybridized with a radio-labelled cDNA for sgp-2. Quantitative analysis using a Fujix Bas 2000 image analyser indicated that in this experiment the steady state level of sgp-2 mRNA was 10.7 times higher in cells cultured on plastic than on the basement membrane matrix.

high over this time period (data not shown). In striking and surprising contrast, the rate of detachment from basement membrane was constant, and low, over the 3 day period, indicating that under identical serum-free conditions, this ECM contributed additional survival signals that were not provided by culture on collagen I or plastic.

The nuclear morphology of the remaining cells showed that apoptotic cells were present within the monolayer cultures on collagen or plastic (Fig. 2b) and supported the conclusion of the time-lapse studies (Fig. 1d). Some of the dying cells were in the centre of confluent islands, indicating that the cells died when still attached both to the substratum and to adjacent cells rather than detaching first and then undergoing apoptosis. Apoptosis in cells that are denied contact with ECM has been termed anoikis (Frisch and Francis, 1994). Since mammary cells undergo apoptosis when attached to collagen I, the mechanism of cell death in this study is distinct from anoikis. To show that culture on EHS matrix suppressed apoptosis and did not merely lead to entrapment of apoptotic cells, we used confocal microscopy to examine the nuclei of cells within three-dimensional structures and found no evidence of apoptosis (Fig. 2c). Furthermore, when equal amounts of DNA were examined for non-random fragmentation, a DNA ladder was present only in cells cultured on collagen or plastic but not in cells cultured on basement membrane (Fig. 2d).

Sulphated glycoprotein-2 (sgp-2, also called TRPM-2) is highly upregulated in the apoptotic, involuting mammary gland (Strange et al., 1992). Northern blotting analysis of mammary epithelial cells plated on tissue culture plastic or on EHS matrix revealed that in the absence of the basement membrane, expression of sgp-2 was markedly increased (Fig. 2e). Thus, although sgp-2 may only be a marker for apoptosis and not involved mechanistically in the control of cell death (French et al., 1994), its expression was upregulated both in vivo and in culture under conditions where apoptosis occurred.

To determine whether the different rates of cell detachment were influenced by the proliferation status of the cells, we assessed the percentage of cells that entered the S phase of the cell cycle during an 8 hour period. Cells on the different substrata were pulsed with bromodeoxyuridine (BrdU) on each of the 3 days following their switch to differentiation medium. Regardless of the nature of the substratum, 95-99% cells did not incorporate BrdU (data not shown). In addition, flow cytometric analysis of the cell cycle phase distribution of cells cultured on either plastic or EHS matrix showed no major differences, with a similar proportion of cells in G_o/G_1 phase (data not shown).

Our results show that mammary epithelial cells can die by apoptosis in culture, but that cell death is suppressed dramatically by basement membrane. The correct type of ECM is required for increased survival, since only a basement membrane matrix, not a collagen I matrix, lowers the rate of apoptosis. Moreover, the survival factor insulin, together with differentiation hormones hydrocortisone and prolactin, does not provide potent enough signals for the suppression of apoptosis in the absence of basement membrane.

Suppression of apoptosis by basement membrane is not mediated by growth factors

We considered it unlikely that survival was mediated by autocrine factors, since fresh medium was added to the cells every 4 hours during the detachment assay. However, EHS

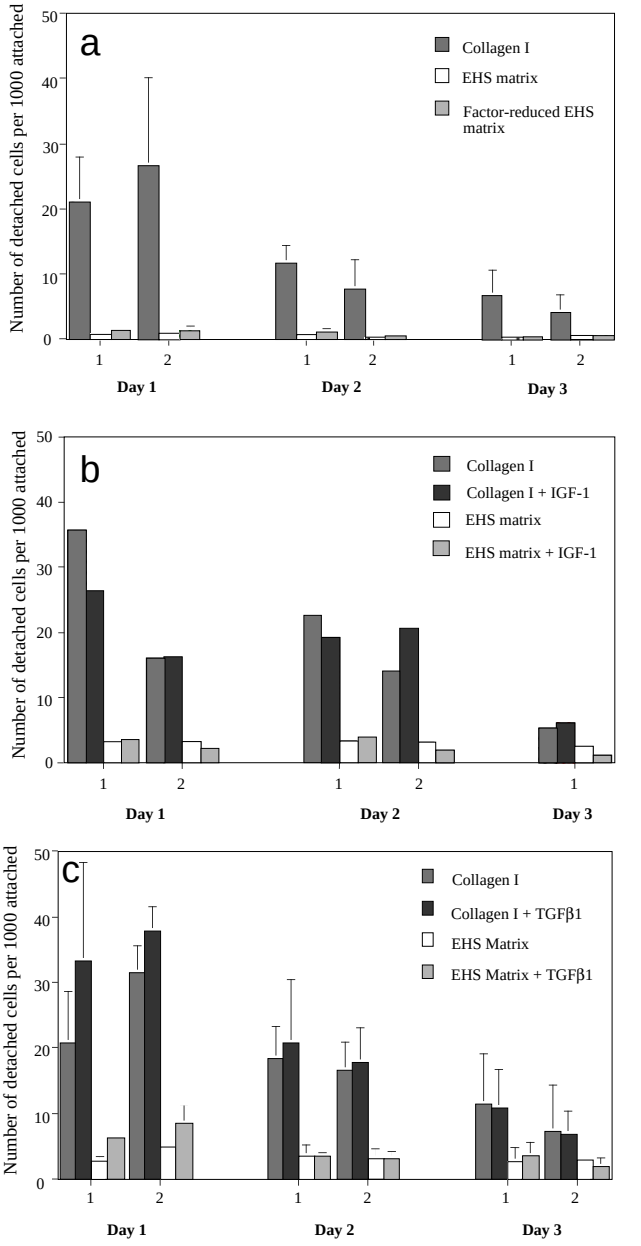

Fig. 3. Suppression of apoptosis by basement membrane was not affected by exogenous growth factors. (a) Detachment assay of cells cultured on collagen I, EHS matrix, or EHS matrix treated with ammonium sulphate to remove most of the exogenous growth factors. The detachment from EHS matrix and the treated matrix preparation were similar, and low, during the 3 day detachment assay. In each case, the data were adjusted to show the number of cells detaching in 4 hours per 1000 cells that initially adhered to the substratum, and (1) and (2) refer to the two different counts on each day of the detachment assay. The results shown were derived from two independent experiments. (b) Detachment assay of cells cultured on collagen I or EHS matrix in the presence of 10 ng/ml IGF-1. Apoptosis in the monolayer cultures on collagen was not suppressed to any major degree by this factor. (c) Detachment assay of cells cultured on collagen I or EHS matrix in the presence of 1 ng/ml TGF-β1. This factor induced a slight increase in apoptosis on day 1 of the detachment assay, but not thereafter. The increased detachment occurred on both collagen and EHS substrata and did not alter the differential in detachment rates. The results were derived from two independent experiments.

Table 1. Basement membrane suppressed apoptosis of individual mammary cells

Time (h)	Substratum	Percentage of apoptotic nuclei		
		Single cells	2-Cell clusters	>2-Cell clusters
0	Collagen I	<1 (1029)	0 (278)	<1 (354)
	EHS matrix	<1 (1066)	<1 (276)	<1 (234)
48	Collagen I	66 (1792)	46 (376)	26 (297)
	EHS matrix	48 (1401)	23 (466)	16 (351)

Primary mammary cells cultured on tissue culture plastic were trypsinised, and single cells and cell clusters were suspended within gels of collagen I or factor-reduced EHS matrix, as described (Streuli et al., 1991). After 2 days of culture in differentiation medium, the gels were fixed, stained with Hoechst 33258, and the numbers of single cells, doublets or clusters of more than 2 cells with normal or apoptotic nuclei were counted. The results are presented in terms of the percentage of total cells that were apoptotic, and the total number of cells counted are indicated in parenthesis. These are the cumulative data from 3 separate experiments, all of which produced similar results.

Table 2. β_1 integrins are required for survival signalling

	Antibody (μg/ml)	Percentage of apoptotic single cells		
		Pre-immune IgG	Anti-β_1 integrin IgG	Fold increase
Expt 1	100	34 (286)	57 (253)	1.7
	200	35 (204)	70 (232)	2.0
Expt 2	100	26 (309)	48 (521)	1.8
	200	28 (207)	61 (475)	2.2

First passage mammary epithelial cells were cultured on EHS matrix in the presence of pre-immune IgG or anti-β_1 integrin IgG for 48 hours, and the numbers of apoptotic single cells were determined. The percentage of apoptotic cells in pre-immune IgG was similar to that in cultures with no antibody present (not shown). The results from 2 separate experiments are presented in terms of the percentage of single cells that were apoptotic, and the total number of cells counted are indicated in parenthesis. Also shown is the fold increase in apoptosis induced by the function-blocking anti-integrin antibody.

matrix contains trace amounts of growth factors that may be responsible for the observed suppression of apoptosis, rather than the matrix per se. We used several approaches to show that this was not likely. First, we compared the rates of cell loss on EHS matrix with a modified preparation of this matrix that had been sequentially precipitated with ammonium sulphate to remove the majority of matrix-bound growth factors. A similar suppression of apoptosis with treated and untreated EHS matrix was observed (Fig. 3a). Second, we examined the effect of one known growth/survival factor that may associate with this matrix, insulin-like growth factor-1 (Vukicevic et al., 1992), and found that it was unable to suppress apoptosis in monolayer culture (Fig. 3b). Third, we added TGF-β1, which induces rapid apoptosis in primary cultures of other epithelial cell types such as hepatocytes (Bayly et al., 1994), and found that although TGF-β1 marginally increased cell detachment from both collagen and EHS matrix, it did not alter the differential in detachment rates from these two substrata (Fig. 3c). Thus, TGF-β1 was unable to overcome the survival signals provided by basement membrane. Fourth, we performed preliminary experiments on mammary cells plated on thick collagen I gels, where endogenous basement membrane is deposited if, and only if, the gel cultures are released into the medium (Streuli and Bissell, 1990). Up to fivefold less cell detachment occurred after gel release, indicating that under otherwise identical conditions, a simple gel manipulation resulting in the formation of a mammary-derived basement membrane reduced the rate of apoptosis (data not shown). Finally, we collected medium from cells cultured for 24 hours on collagen I or on EHS matrix, then cleared the medium of cell debris and placed it on cells cultured on the alternative matrix; there were no changes in the rates of cell detachment in comparison with controls, indicating that autocrine factors were not likely to be responsible for cell death or survival (data not shown). Together, these data suggest that any major role for exogenous growth factors in the ECM-mediated regulation of mammary epithelial cell survival is unlikely. This was further corroborated in experiments described below.

Direct action of basement membrane as a survival factor

Mammary cells cultured on collagen or plastic interact with each other through adherens junctions and desmosomes, and are polarised, since the tight junction antigen ZO-1 is exclusively located on the apical cell surface (C.H.S., unpublished observations). However, as demonstrated above, such interactions were not sufficient to suppress apoptosis (Figs 1 and 2). Following culture on basement membrane, however, the cells change their morphology and exhibit a higher degree of cell-cell communication and polarity (Aggeler et al., 1991). It was therefore important to assess whether the survival stimulus provided by basement membrane was mediated through an increased extent of intercellular adhesion and polarity, or was provided directly by the matrix itself.

To answer this question, we utilised a previously published assay to examine the behaviour of single mammary cells and small clusters of cells cultured within physiological substrata (Streuli et al., 1991). First passage cells were suspended inside gels of collagen I, or in ammonium sulphate-treated EHS matrix. We had already shown that single cells cultured under these conditions were non-polarised and unable to interact with other cells (Streuli et al., 1991). After culture in differentiation medium for 48 hours, the numbers of single cells or small cell clusters with normal or apoptotic nuclei were counted (Table 1). Many of the single cells suspended in collagen underwent apoptosis, but the basement membrane matrix retarded cell death. To confirm that cell-matrix interactions were required for survival, mammary cells were cultured on EHS matrix in the presence of a function-blocking anti-β_1 integrin antibody (Table 2). Up to 2.2 times as many single cells underwent apoptosis in the presence of this antibody as opposed to control IgG. Thus the basement membrane matrix provides direct survival signals that can be transduced through integrins.

We also observed that there were fewer apoptotic nuclei in small groups of cells cultured within either collagen or EHS gels, indicating that cell-cell contacts also reduced the levels of apoptosis (Table 1). It has already been shown that small clusters of cells cultured under these conditions in the collagen matrix can deposit basement membrane proteins (Streuli et al., 1991), so it is possible that this endogenous matrix contributed to enhanced survival in cell clusters. Together, our data show that basement membrane contributes directly to the suppression of apoptosis of single cells, but optimal survival requires additional signals generated by cell-cell adhesion.

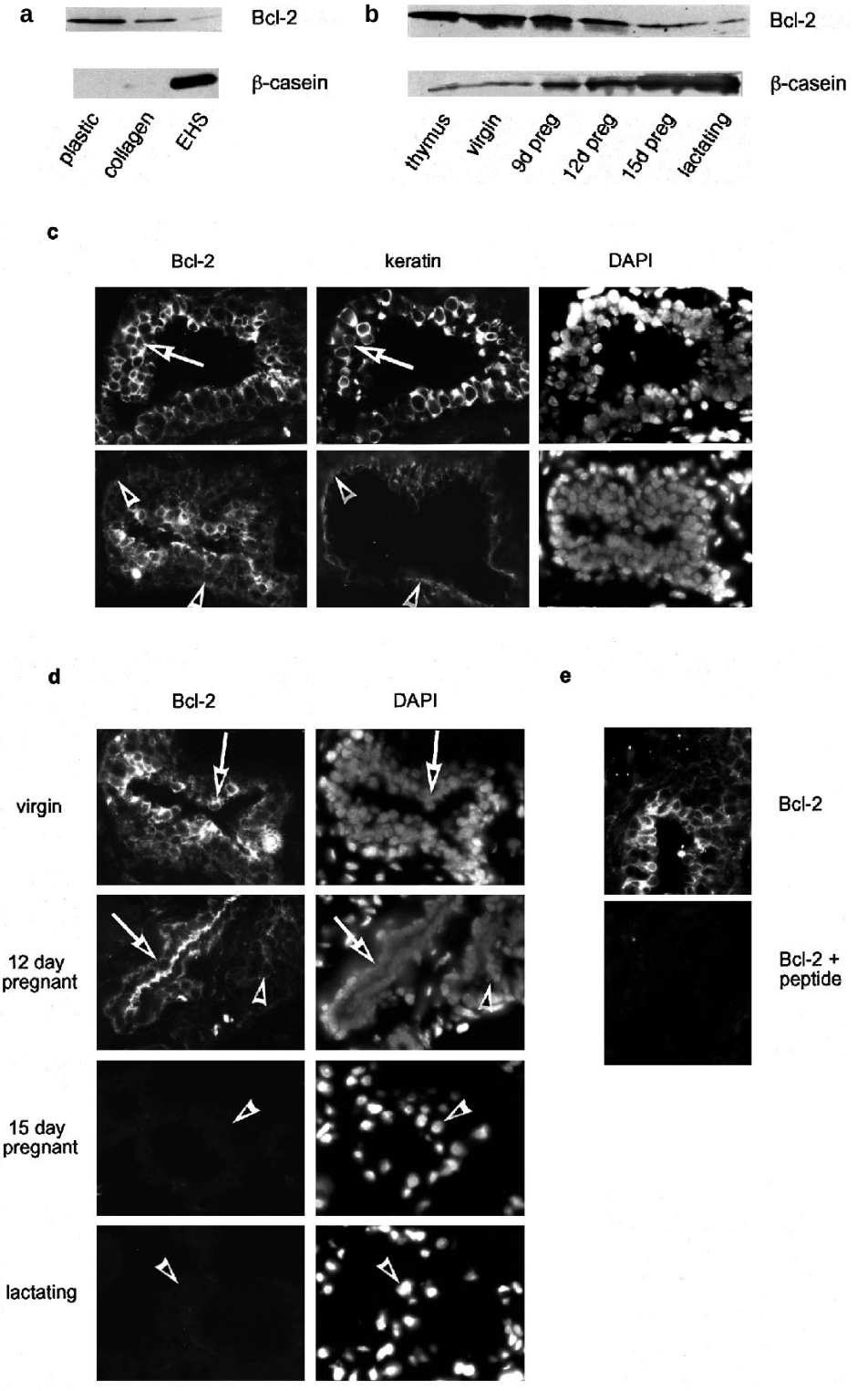

Fig. 4. Bcl-2 expression in mouse mammary epithelium. (a) Matrix dependence of Bcl-2 expression in culture. Western blots of Bcl-2 and the differentiation antigen, β-casein, in mammary cells cultured for 3 days in differentiation medium on tissue culture plastic, collagen I, and EHS matrix. Equal samples of total cell protein were separated by SDS-PAGE, transferred to nylon membranes, and probed with antibodies as described in Materials and Methods. Identical gels were stained with Coomassie Brilliant Blue to confirm equal loading of protein (not shown). Control experiments with excess peptide confirmed the specificity of the Bcl-2 reaction. (b) Modulation of Bcl-2 expression in vivo. Western blots of total protein extracted from mammary gland at different stages of pregnancy and from thymus gland were probed with antibodies specific for Bcl-2 or for the milk protein, β-casein. Identical gels were stained with Coomassie Brilliant Blue to confirm equal loading of protein, and control experiments with excess peptide confirmed the specificity of the Bcl-2 reaction (not shown). Note that the levels of Bcl-2 diminish during pregnancy and are virtually absent at lactation. β-casein levels increase to a maximun at lactation. (c) Bcl-2 is expressed in luminal epithelial cells of mammary gland ducts. Cryosections of non-pregnant mammary gland were double stained by indirect immunofluorescence with a rabbit anti-Bcl-2 peptide antiserum (left panels) and mouse monoclonal antibodies to cytokeratins (centre panels), then counterstained with DAPI (right panels). In the upper series, anti-cytokeratin 8 (LE41) was used to localise luminal epithelial cells, and in the lower series anti-cytokeratin 14 (LL001) was used to localise myoepithelial cells. Note that Bcl-2 mostly co-distributed with luminal epithelial cells (arrow). Myoepithelial cells were generally negative for Bcl-2, but low level staining was sometimes apparent. Bcl-2 staining was not present in stromal cells. (d) Expression of Bcl-2 disappears in alveolar epithelium during pregnancy and lactation. Cyrosections of non-pregnant, pregnant, and lactating mammary gland were stained by indirect immunofluorescence with a rabbit anti-Bcl-2 peptide antiserum (left panels) and counterstained with DAPI (right panels). Bcl-2 was expressed in ducts of non-pregnant and pregnant mammary gland (arrows in virgin and 12 day pregnant gland). As alveoli formed, the level of Bcl-2 staining was reduced (arrowhead in 12 day pregnant gland). In mature alveoli of 15 day pregnant and lactating mammary gland, Bcl-2 staining was absent (arrowheads). For the Bcl-2 micrographs, exposure conditions onto both negative film and photographic paper were identical in each case. (e) Specificity of staining with the anti-Bcl-2 antibody. The DHA-7 anti-Bcl-2 peptide antibody, which was raised against amino acid sequence 41-54 of mouse Bcl-2, was used for all these immunostaining experiments. Excess peptide was included in some experiments to confirm specificity of staining. This experiment shows two serial sections of non-pregnant mammary gland immunostained either with the Bcl-2 antibody (upper panel) or with the antibody incubated with excess peptide (lower panel). Exposure conditions onto both negative film and photographic paper were identical.

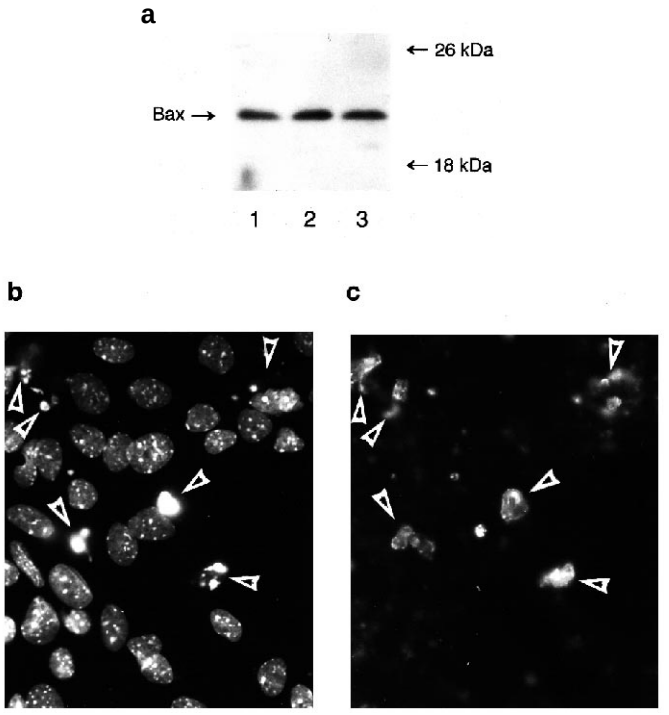

Fig. 5. Bax expression in cultured mammary epithelium. (a) Matrix dependence of Bax protein expression. Western blots of Bax in mammary cells cultured for 1 day in differentiation medium on (1) EHS matrix and (2) collagen I. Similar results were obtained after culture for 3 days in differentiation medium (not shown). As a control for the Bax reaction, an aliquot of a cell lysate isolated from M1 myeloid leukemia cells was included (lane 3). Equal samples of total cell protein were separated by SDS-PAGE, transferred to nylon membranes, and probed with antibodies as described in Materials and Methods. Identical gels were stained with Coomassie Brilliant Blue to confirm equal loading of protein (not shown). Control experiments with excess peptide confirmed the specificity of the Bax reaction. (b,c) Bax is expressed at high levels in the apoptotic population of cells cultured on collagen I. Monolayers of first passage mammary cells were cultured overnight in differentiation medium, then fixed and processed for immunofluorescence with anti-Bax antiserum. The cells were counterstained with Hoechst 33258 to localize apoptotic cells. Only the apoptotic cells showed high levels of Bax staining (arrowheads). Control experiments with excess peptide showed no staining. Additional experiments performed with cytospins of cells that had detached overnight, or of washed monolayers that were trypsinised, confirmed that Bax staining was visible only in apoptotic cells but not in viable cells; in addition, Bax was not present in trypsinised cells recovered from EHS cultures (not shown). Explanations for the discrepancy between the western blot and fluorescence data are that only a small percentage of cells are apoptotic at any one time, so increased levels of Bax would not be detectable by western blot, or alternatively that Bax is redistributed in dying cells so that the antibody epitope in live cells is masked but in apoptotic cells is exposed.

Involvement of Bcl-2 and Bax in mammary apoptosis

The mechanism by which survival factors suppress apoptosis is not well understood. One intracellular pathway for the suppression of apoptosis is that mediated by Bcl-2. To determine whether Bcl-2 might be involved in the matrix-dependent control of survival, we examined the levels of Bcl-2 in our cultures. It was present in first passage mammary cells, but was expressed more highly in cells cultured on collagen I or plastic than on the basement membrane matrix (Fig. 4a). Since these findings were counter-intuitive, we examined the distribution of Bcl-2 in mammary gland in vivo. It was expressed strongly in ductal luminal epithelial cells, which in the mouse do not contact a basement membrane, and was either absent or at very low levels in myoepithelial cells and in the outlying stroma (Fig. 4c). However, in alveoli of day 12 pregnant mice where the epithelial cells interact directly with basement membrane, there was less Bcl-2, and by day 15 of pregnancy and during lactation, Bcl-2 staining was absent (Fig. 4d). These results were echoed by a reduction in the overall levels of Bcl-2 protein in mammary tissue during pregnancy (Fig. 4b). Thus, in culture and in vivo the levels of Bcl-2 were lowest in cells in direct contact with a basement membrane, suggesting that this type of ECM provides dominant survival signals that are independent of Bcl-2.

Since Bcl-2 itself did not appear to be responsible for matrix-mediated survival, we examined the expression of Bax, a binding partner for Bcl-2. Our initial analysis by western blotting showed that the total levels of Bax protein remained similar in cells cultured on different substrata (Fig. 5a). However, a detailed immunofluorescence study revealed that Bax was highly expressed in individual dying cells cultured on collagen I, but not in cells containing viable nuclei (Fig. 5b,c). Bax was additionally present in apoptotic but not viable cells released from monolayers by trypsinisation, and also in apoptotic but not healthy M1 myeloid leukaemia cells (data not shown). Since Bax expression was also upregulated during apoptosis of mammary cells in the involuting gland (A. Metcalfe, J. A. Hickman and C. H. Streuli, unpublished data), we conclude that Bax may have a central role in mediating apoptosis both in vivo and in culture, and might therefore be a target for regulation by the ECM.

DISCUSSION

This study demonstrates that basement membrane is required for the survival of early passage epithelial cells isolated directly from mammary gland. It has been proposed that the engagement of apoptosis is a default process and to avoid death cells must receive signals to survive (Raff, 1992). Here we show that soluble trophic factors are not the only environmental arbiters of life and death in mammary epithelium, but that an interaction with a specific type of ECM, basement membrane, also confers potent signals for survival.

We observed that mammary epithelial cells attached and spread on physiological ECM such as collagen I, but subsequently died by apoptosis despite culture in medium containing lactogenic hormones, a condition that induced survival on basement membrane. Apoptosis took place asynchronously, resulting in a gradual disappearance of individual cells from the monolayer over several days, rather than detachment of contiguous sheets of cells. Three lines of evidence demonstrated that the cells died by apoptosis: the ‘dance of death’ observed by time-lapse videomicroscopy, the changes in nuclear morphology, and the internucleosomal cleavage of DNA. Previous studies have indicated that the complete

absence of matrix, by culture on denatured agarose or non-adhesive polyhydroxyethylmethacrylate, triggers rapid apoptosis in endothelial and epithelial cells (Meredith et al., 1993; Frisch and Francis, 1994). This form of programmed cell death, induced when cell attachment to a substratum is prevented, has been termed anoikis (Frisch and Francis, 1994). However, the death we describe here differs from anoikis, since the presence of apoptotic nuclei observed in cells still attached to the culture dish, together with our time-lapse analysis, showed that death occurred before, rather than after, detachment from the substratum. An apoptotic morphology in attached epithelial cells seems to be a characteristic of primary cultures, at least for mammary cells and hepatocytes (Bayly et al., 1994), and contrasts with tumour cell lines derived from these tissues, where only detached cells exhibit condensed and fragmented nuclei (Bayly et al., 1993; Wilson et al., 1995).

Epithelial cells in mammary alveoli do not normally interact with stromal ECM but instead contact a basement membrane. Our experiments showed that adhesion and interaction with a basement membrane matrix in culture protects them against cell death, but that a plastic or collagen I matrix was unable to support survival. Thus mammary cell survival was controlled by a specific type of cell-matrix interaction, and in the absence of a correct ECM the cells died by apoptosis. The inference from our study is that basement membrane has an important role as a survival factor for normal mammary epithelial cells in vivo. This survival function may be critical for directing accurate tissue morphogenesis during development if cells migrating away from their own microenvironment are deleted by apoptosis, and for maintaining tissue homeostasis during adult life. Conversely, a deregulated contact with the ECM may also be involved in the overall cell gain that occurs in breast cancer. One molecular mechanism that may contribute to mammary carcinogenesis and metastasis, for example, might be the abrogation of a requirement for cell adhesion to basement membrane for suppressing apoptosis (Howlett et al., 1995).

Continued active signalling by basement membrane is essential in the mammary gland for differentiation and transcription of milk protein genes (Streuli, 1995). Our results demonstrate that this same environmental cue also generates signals for survival. These signals are to some extent direct, since a function-blocking anti-β_1 integrin antibody doubled the rate of apoptosis in single cells cultured with the basement membrane matrix. Thus in mammary gland, ligation of integrin receptors may be required to prevent a default apoptotic process. Integrins have been shown to be required for the survival of other cell types, and α_v integrin in particular has been shown to have a prominent role. This subunit is required for survival of melanoma cells cultured in the absence of exogenous growth factors (Montgomery et al., 1994), and function-blocking anti-α_v integrin antibodies inhibit angiogenesis and cause endothelial cells, which would otherwise proliferate and form new capillaries, to undergo apoptosis instead (Brooks et al., 1994). We have previously shown that functional β_1 integrins are also required for matrix-control of differentiation (Streuli et al., 1991). Although the spectrum of integrins on mouse mammary cells cultured on plastic and EHS matrix are similar, with α_2, α_3, β_1 and β_4 being the major subunits (Delcommenne and Streuli, 1995), it is possible that the intracellular signals for differentiation and survival are generated through alternative integrin heterodimers.

In addition to cell-matrix interactions, cell-cell contact is important for survival of epithelial cells. This has been demonstrated in other systems such as the LM1863 colon carcinoma line, where intercellular adhesion is required to prevent apoptosis, although interestingly in this case α_v integrins provide the cell adhesion-mediated survival signal (Bates et al., 1994). Our experiments showed that although basement membrane suppressed apoptosis of single cells embedded within the matrix to a greater extent than collagen I, the differential in survival was not as great as for cells plated on top of these substrata. This suggests that cell-cell contacts also contribute to mammary epithelial survival, and is supported by the observation that small clusters of cells cultured within ECM gels were better able to survive than isolated single cells. However, additional signals to those provided solely by cell-cell adhesion must be necessary, as cells within confluent monolayers on collagen or plastic still underwent apoptosis. We therefore suggest that a combination of signals from both cell-matrix and cell-cell adhesions are required above a certain threshold level to prevent irreversible commitment to programmed cell death.

Even though survival is a prerequisite for functional differentiation, it is likely that some of the intracellular pathways protecting cells against apoptosis are different from those that trigger differentiation. For example, prolactin-derived signals cooperate with laminin to activate transcription factors that regulate milk protein gene transcription (Streuli et al., 1995b). But we have preliminary data showing that prolactin is not necessary for basement membrane-mediated survival, so it is likely that the signals for differentiation and survival are separable (data not shown). One of the best studied, but least understood, routes controlling apoptosis is that mediated by the survival gene Bcl-2 and its related proteins (Oltvai and Korsmeyer, 1994). Bcl-2 was expressed in the luminal epithelial cells of mammary ducts, but not in alveoli. Although alveolar cells contact basement membrane, most of the ductal cells in mouse mammary gland do not, as there is a layer of myoepithelium separating them from the ECM. Bcl-2 was also present in cultured mammary epithelium, but cells surviving on a basement membrane matrix expressed only low levels of Bcl-2 compared to those cells cultured as a monolayer on collagen I or on plastic. Since Bcl-2 promotes survival, our data from both culture and in vivo studies suggest that Bcl-2 and basement membrane may contribute independently to mammary epithelial survival, with Bcl-2 being dominant in ductal luminal cells, and basement membrane providing the primary survival signal in alveolar cells that are destined to apoptose at the end of their differentiation period.

Since Bcl-2 did not appear to mediate basement membrane-mediated survival, we asked whether the levels of Bax correlated with mammary apoptosis either in culture or in vivo. Bax shares homology with Bcl-2, but high levels of Bax favor cell death in the absence of extracellular survival signals (Oltvai and Korsmeyer, 1994). In cultured mammary cells, the amount of total Bax protein was not altered by the substratum, but immunofluorescence revealed that individual dying cells in monolayer contained higher levels of Bax protein. We also found that culture on collagen I or plastic resulted in the total *bax* mRNA levels being elevated (data not shown), but since

Bax is a cell death regulator, its protein turnover rate would be expected to be rapid thus explaining any differential in total protein and mRNA levels. The Bax promoter contains several Sp1 transcription factor recognition motifs (Miyashita and Reed, 1995), so our recent observation that the DNA-binding activity of Sp1 is much higher in cells cultured on collagen I or plastic than EHS matrix (Streuli et al., 1995b) may provide a mechanistic explanation for increased Bax expression on these substrata. The studies on Bax in culture are supported by our finding that *bax* mRNA and Bax protein were more abundant in the apoptotic, involuting gland than in mammary tissue at other times of development, and together they indicate that Bax may have a critical, though not necessarily exclusive, role in regulating mammary apoptosis.

A study was recently published that indicated that cell death can also result following culture of a mammary epithelial cell line in the absence of basement membrane (Boudreau et al., 1995). It was suggested that interleukin-1β converting enzyme (ICE), thought to act downstream or in parallel with the Bcl-2/Bax survival checkpoint (Oltvai and Korsmeyer, 1994), was involved in this process. However, it is now more likely that homologues of ICE may be involved, rather than ICE itself, since mice that are homozygous null for ICE still progress through mammary involution and undergo apoptosis normally (Li et al., 1995). Taken together, our studies and those of Boudreau et al. (1995), indicate that in mammary cells several regulators of apoptosis are controlled by interactions with the ECM.

Since apoptosis occurs naturally in the involuting mammary gland, the cell death we observed in culture may reflect the engagement of a normal apoptotic pathway triggered by the loss of correct ECM. Within 2 days of weaning, epithelial cells begin to detach from the alveolar layer of cells and underlying basement membrane, and undergo apoptosis. At 4 days, matrix-metalloproteinase expression is maximal, degradation of the basement membrane occurs, and the gland undergoes catastrophic apoptosis (Martinez-Hernandez et al., 1976; Strange et al., 1992; Talhouk et al., 1992). Evidence for a causal role of ECM as a survival factor in vivo comes from studies with transgenic mice that express ectopic stromelysin-1 under the control of a milk protein promoter (Sympson et al., 1994). In these mice, active stromelysin-1 was inappropriately expressed during mid to late pregnancy, at which time 10-15% of epithelial cells within the mammary gland were apoptotic (Boudreau et al., 1995). The mammary cells from pregnant mice that we isolated for our studies were destined to die at the end of their lactational period. However, in removing cells from their natural environment and placing them on plastic or collagen I, an apoptotic process that would normally occur only at weaning has been artificially brought forward. We therefore propose that our culture model mimics the process of mammary gland involution occurring in vivo.

In conclusion, the specialized ECM, basement membrane, provides an essential contribution toward the survival of mammary epithelium. Suppression of the apoptotic program is widely regarded as being dependent on the presence of extracellular factors, although most attention has been given to the role of growth factors and cytokines. Our work using mammary epithelial cells now indicates that basement membrane provides direct signals that should be considered as part of a repertoire of triggers required for maintaining the viability of normal adherent cells.

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