Suppression of ICE and Apoptosis in Mammary Epithelial Cells by Extracellular Matrix

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Apoptosis (programmed cell death) plays a major role in development and tissue regeneration. Basement membrane extracellular matrix (ECM), but not fibronectin or collagen, was shown to suppress apoptosis of mammary epithelial cells in tissue culture and in vivo. Apoptosis was induced by antibodies to I?1 integrins or by overexpression of stromelysin-1, which degrades ECM. Expression of interleukin-1 p converting enzyme (ICE) correlated with the loss of ECM, and inhibitors of ICE activity prevented apoptosis. These results suggest that ECM regulates apoptosis in mammary epithelial cells through an integrin-dependent negative regulation of ICE expression.

Growth, differentiation, and apoptosis are alternative cellular pathways that are each crucial to normal development and the establishment of tissue-specific function. Like growth and differentiation, apoptosis requires active and coordinated regulation of specific genes. In mammalian cells, these genes include BCL-2, a homolog of the Caenorhabditis elegans ced-9 gene, which is a potent suppressor of death (1), and ICE, a homolog of the ced-3 gene, which can actively kill cells (2). The products of the BCL-2 and ICE genes also appear to function like their C. elegans counterparts (1-3).

The nature of the ECM can influence the apoptotic program in mammalian cells. Establishment of mammary gland alveolar morphology and expression of milk-specific genes are absolutely dependent on deposition of a laminin-rich ECM (4). In addition, involution of the gland, which follows expression of the lactational phenotype, is characterized by degradation of this ECM by metalloproteinases (5) and is accompanied by apoptosis (6, 7). Cell attachment, mediated by integrin-ECM interactions, can suppress apoptosis in shortterm two-dimensional cultures for up to 30 hours (8).

To determine whether ECM regulates apoptosis, we compared the response of CID-9 mammary epithelial cells (MECs) plated directly on tissue culture plastic, in the absence of serum, with those plated on an exogenous basement membrane ECM. Unlike plastic, fibronectin, or type I collagen, this Englebreth-Holm-Swarm (EHS) matrix directs the cells to differentiate, as manifested by the formation of three-dimensional alveolar structures and expression of milk proteins (9). After 4 to 5 days on plastic, despite strong adhesion and spreading, the cells began to display characteristics of apoptosis including nucleosomal DNA laddering (Fig. 1A), expression of the apoptosis-associated gene SGP-2 (7) (Fig. 1B), and nuclear condensation (Fig. 1C). In situ analysis revealed that fragmented DNA was present in 10 to 20% of cells (Fig. iD). In contrast, the cells plated on ECM did not display these apoptotic features for up to 10 days (Fig. 1, A, B, and E). Similar results were observed when the ECM was pretreated with ammonium sulfate to remove growth factors (10). To eliminate the possibility that suppression of apoptosis was due to residual growth factors in the EHS matrix, we plated MECs on porous filters (11), whereupon the cells deposited their own basement membrane. This endogenous basement membrane also suppressed apoptosis in long-term cultures (Fig. iF).

To demonstrate that ECM-derived signals suppress apoptosis, we disrupted cell-ECM interactions by addition of an antibody to β_1 integrin (12). Two days after addition of this antibody, a substantial increase in nucleosomal DNA laddering was observed, even in cells still adherent to their endogenous matrices (Fig. 1G). MECs that were attached to culture dishes coated with either fibronectin or type I collagen displayed a degree of apoptosis similar to MECs

cultured on plastic (Fig. 1H), indicating that suppression of apoptosis required an intact basement membrane ECM.

To determine whether proteolytic destruction of an existing basement membrane could induce apoptosis, we established a culture model of mammary gland involution. CID-9 cells were cotransfected with an inducible expression vector encoding a stromelysin-1 autoactivating mutant under control of the Rous sarcoma virus (RSV) promoter linked to a lac repressorbinding intron and with a vector encoding the lac repressor-binding protein (13). MECs were cultured on filters for 3 days, and stromelysin-1 expression was induced by addition of 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Within 72 hours, there was a substantial increase in apoptosis-associated DNA laddering in the cells expressing stromelysin-1 but not in the uninduced controls (Fig. 2A). Apoptosis depended on proteolytic activity of the stromelysin-1 because it was inhibited by the addition of the metalloproteinase inhibitor GM6001 (14).

We then examined apoptosis in vivo in transgenic mice expressing the stromelysin-1 gene under control of the whey acidic milk protein promoter (15), which is activated in mid- to late pregnancy. DNA anal-ysis revealed that unlike the MECs in normal animals (Fig. 2B), at least 10 to 15% of MECs in the transgenics were apoptotic in midpregnancy (Fig. 2C). Thus, degradation of ECM by stromelysin-1 results in apoptosis both in culture and in vivo.

To determine whether apoptosis of MECs was mediated by ICE, a known inducer of apoptosis in mammalian cells, we transfected CID-9 cells with a vector encoding crmA, a viral gene product that specifically inhibits the enzymatic activity of ICE (16). The crmA transfectants showed an 80% reduction in apoptosis-associated DNA laddering compared to control cells (Fig. 3, A and B). We also treated cells plated on plastic with BACMK, an inhibitor directed at the active site of ICE (17). BACMK reduced DNA laddering in CID-9 cells by up to 80% after 5 days as compared to uninhibited controls (Fig. 3B). These results indicate that in the absence of ECM, apoptosis of MECs occurs largely through the activity of ICE.

We also investigated the expression of ICE in the mammary gland in vivo. The 1.6-kb ICE mRNA (18) was not expressed in the lactating gland but was induced during involution (Fig. 4A), when apoptosis occurs in this tissue (7). To determine whether the regulation of ICE expression was directly related to the presence of ECM, we examined ICE mRNA expression in CID-9 cells. CID-9 cells cultured on plastic contained large amounts of ICE mRNA and the 45-kD ICE precursor protein and enzymatically active 20-kD subunit (19), whereas those plated on ECM contained little or no ICE mRNA or protein (Fig. 4, B and C).

We conclude that three-dimensional ECM, acting through integrin receptors, not only directs committed MECs to establish and maintain the differentiated state but also suppresses the expression of ICE and prevents apoptosis. Consequently, the proteolytic degradation of ECM such as occurs during mammary gland involution leads to the loss of the differentiated state, induction of ICE expression and activity, and ultimately apoptotic cell death both in vivo and in culture. Although our data cannot distinguish between ICE and as yet unidentified ICE gene family members that might also be blocked by active site-directed inhibitors or recognized by antibodies to ICE, we show that survival requires not only adhesion, but also specialized P integrin-mediated signals derived from specific ECM components. The nature of these signals

and their ability to modulate the expression of ICE remain to be elucidated, as do the in vivo substrates for ICE or related enzymes and the mechanism or mechanisms by which they influence cell death.

References and Notes

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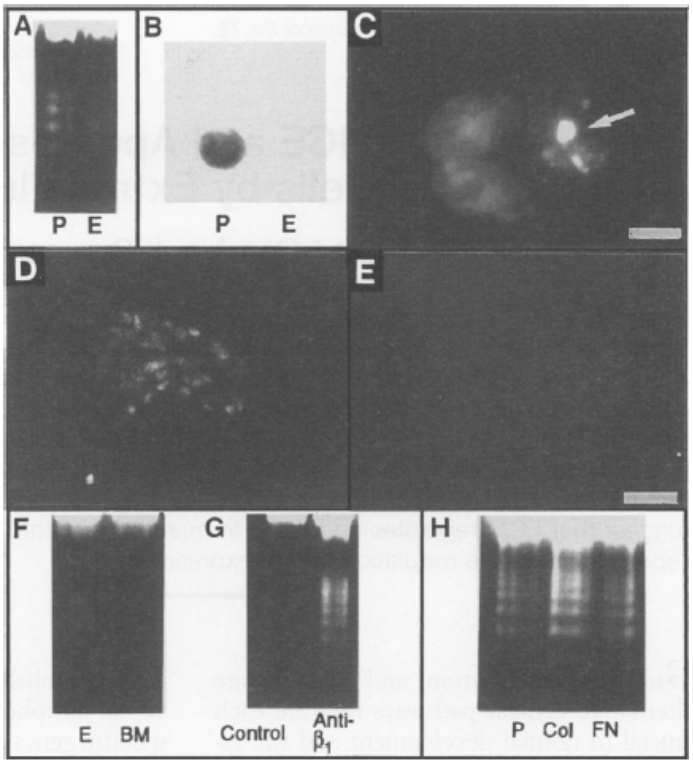
9. CID-9 mammary epithelial cells [C. Schmidhauser, C. A. Myers, G. F. Casperson, M. J. Bissell, Proc. Natl. Acad. Sci. U.S.A. 87, 9118 (1990)] were cultured in Dulbecco's modified Eagle's medium-F-1 2 without serum and supplemented with insulin (5pug/mI), hydrocortisone (1 Lg/mI), and prolactin (3 ptg/ml) at a density of 3.8 x 106 cells per 100 mm plastic culture plates for 4 to 10 days. When indicated, plates were precoated with 1 to 2 ml of exogenous ECM prepared from the mouse EHS [H. Kleinman et al., Biochemistry 26, 312 (1986)]. Mouse SGP-2 mRNA was detected with a 1.4-kb complementary DNA (cDNA) probe. DNA integrity was assessed by nucleosomal laddering (7), or by DNA fragmentation in individual cells visualized with the Apoptag kit (Oncor, Gaithersburg, MD) or with the TUNEL method [Y. Gavrieli, Y. Sherman, S. A. Ben-Sasson, J. Cell Biol. 119, 493 (1992)]. Chromatin condensation was detected by staining with acridine orange (10 gg/mI) dissolved in phosphate-buffered saline.
10. M. Taub, Y. Wang, T. Szczesny, H. K. Kleinman, Proc. Natl. Acad. Sci. U.S.A. 87, 4002 (1990); the faint ethidium bromide staining observed in DNA extracted from cells cultured on EHS is attributable to randomly degraded DNA in the initial EHS preparations, which is removed after the ammonium sulfate precipitation.
11. CID-9 cells were cultured on Milli-Cell CM filters (Millipore) under conditions that promote synthesis and vectorial secretion of milk proteins [G. Parry, B. Cullen, C. S. Kaetzel, R. Kramer, L. Moss, J. Cell Biol. 105, 2043 (1987); E. Reichmann, R. Ball, B. Groner, R. R. Friis, ibid. 108,1127 (1989)]. The cells were then lysed hypotonically and removed [R. H. Kramer, G. M. Fuh, K. G. Bensch, M. A. Karasek, J.Cell Physiol. 123, 1 (1985)]. By immunohistochemical analysis we detected an insoluble, basally deposited ECM rich in type IV collagen and laminin.
12. A rabbit polyclonal antibody to guinea pig P1, integrin [C. H. Damsky, K. A. Knudson, C. A. Buck, J. Cell. Biochem. 18, 1 (1982)] was added at a dilution of 1:200 for 2 days to MECs that had been cultured for 2 days on filters. Normal rabbit serum was used as a control.
13. The pOPRSVM2 vector was constructed by insertion of a 1.2-kb cDNA fragment encoding an autoactivating stromelysin-1 mutant [R. Sanchez-Lopez, R. Nicholson, M. C. Gesnel, L. Matrisian, R. Breathnach, J. Biol. Chem. 263,11892 (1988)] designated M2 (14) into the Not site of the pOPRSV vector. This vector contains RSV long terminal repeat (LTR) sequences

upstream of an intron sequence with lac repressor binding sites. The pOPRSVM2 vector was cotransfected with the p3SS' vector encoding the lac operon repressorbinding protein (Lacswitch System, Stratagene).
14. The GM6001 peptide metalloproteinase inhibitor [D. Grobelny, L. Poncz, R. E. Galardy, Biochemistry 31, 7152 (1992)] was used at a concentration of 10 [uM, added daily over a period of 5 days. 15. C. J. Sympson et al., J. Cell Biol. 125, 681 (1994).
16. A 1.4-kb cDNA of crmA, p996 [C. A. Ray et al., Cell 69, 597 (1992)], was inserted into the Eco RI site of pBluescript KS (Stratagene). The cDNA was excised as a Xba l-Kpn fragment and inserted into the Nhel-Kpn sites of the pBK-RSV expression vector under the control of the RSV LTR. This vector was transfected into CID-9 cells.
17. Boc-aspartyl (benzyl) chloromethylketone (BACMK) penetrates cells and binds covalently to the active site of ICE. At a concentration of 2.5 [LM, this inhibitor reduces ICE-mediated processing of mature interleukin-1, in a monocytic cell line by 85% without affecting tumor necrosis factor processing (R. Black, unpublished data). Fresh medium containing 0 to 5.0 gM BACMK dissolved in dimethyl sulfoxide was added daily on days 2 to 5 in cultures of CID-9 cells. These concentrations did not produce notable cytotoxic effects or reduce functional differentiation and expression of P-casein in the MECs (N. Boudreau, C. J. Sympson, Z. Werb, M. J. Bissell, data not shown). Inactivated BACMK or tosyl-asparginine-methyl ester (Sigma) (2 to 5 [uM) in dimethyl sulfoxide was used as a control (N. Boudreau, C. J. Sympson, Z. Werb, M. J. Bissell, data not shown). DNA and RNA were isolated 4 hours after the final treatment.
18. ICE mRNA was detected by hybridization of the blots with a 32P-labeled 1.2-kb Eco RI fragment of murine ICE cDNA plasmid J.348. Blots were hybridized for 16 hours at 42°C, washed in 0.1 % SDS-0.1 x standard saline citrate at 68°C, and exposed to film for 3 days at -70°C.
19. Cells were cultured under serum-free conditions for 5 days and directly lysed into SDS-Laemmli sample buffer to prevent artifactual activation of the proenzyme. Lysates from 1 x 105 cells were subjected to electrophoresis on 12.5% SDS-polyacrylamide gels, and the separated proteins were transferred to nitrocellulose membranes. Membranes were blotted with a 1:300 dilution of rabbit antiserum to polyclonal ICE [N. A. Thornberry et al., Nature 356, 768 (1992)] and visualized with enhanced chemiluminescence (Amersham). Control lysates from 1 x 105 crmA-transfected CID-9 cells display the 45-kD precursor protein but not the processing intermediates or the 20-kD active subunit (N. Boudreau, C. J. Sympson, Z.Werb, M. J. Bissell, data not shown).
20. We thank R. Strange for the SGP-2 cDNA probe, C. Damsky for antisera to l3, integrin, R. Galardy for the GM6001, J. Yuan forthe ICEcDNA, D. Pickup for the crmA cDNA, R. Black for sharing unpublished data and for the BACMK, and S. Molineaux for antisera to ICE. Supported by the National Cancer Institute (grant CA 57621), the U.S. Department of Energy, Office of Health and Environmental Research (contracts DE-AC-03-76-SF00098 and DE-AC-03-76-SF10102), the Medical Research Council of Canada, and the National Institute of Environmental Heatth Sciences (National Research Service Award ES 07106).

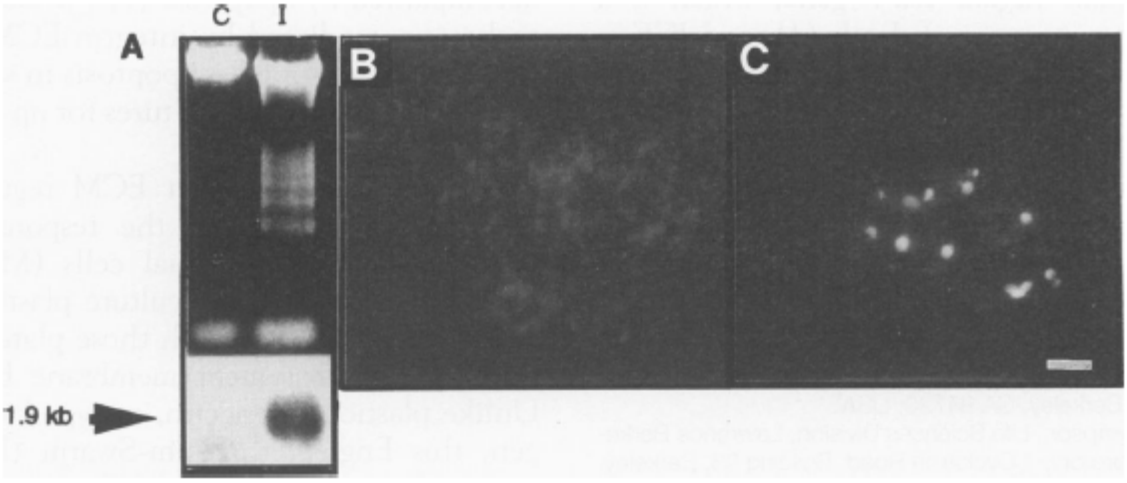
Figures

FIGURE 1



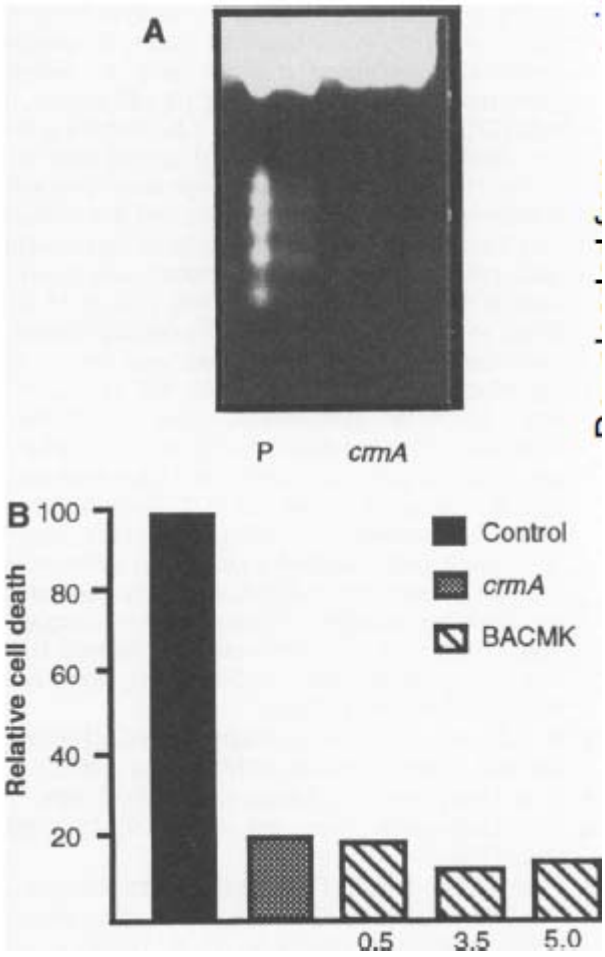
Characteristics of apoptosis in CID-9 cells. (A) Electrophoretic analysis of total DNA (20 μg) from cells cultured for 7 days on plastic (P) or EHS basement membrane (E). (B) RNA blot hybridized with a probe that detects the 2.4-kb mRNA for the apoptosis-associated gene SGP-2. (C) Acridine orange staining of cultured cells on plastic (arrow points to apoptotic cells). Scale bar, 58 μm. In situ analysis of DNA fragmentation in individual cells cultured on plastic (D) or EHS basement membrane (E) detected by fluorescein isothiocyanate-digoxigenin nucleotide labeling of 3'-OH DNA ends (Apoptag, Oncor). Scale bar, 90 μm. Electrophoretic analysis of total DNA (20 μg) from CID-9 cells (F) cultured on EHS (E) or allowed to form endogenous basement membrane (BM) for 5 days, (G) treated with normal rabbit serum (control) or anti-β_1 integrin for 2 days, or (H) cultured on plastic (P), type collagen (200 μg/ml) (Col), or fibronectin (50 μg/ml) (FN) for 5 days.

FIGURE 2



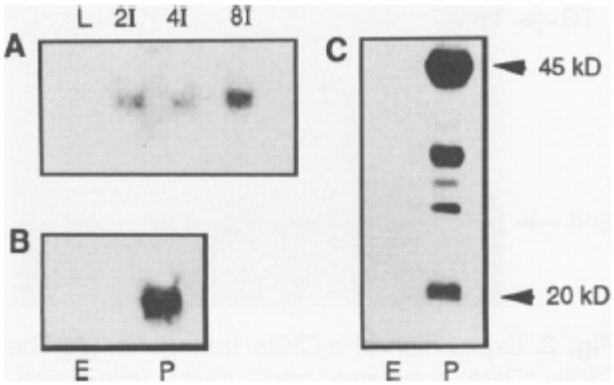
Apoptosis in cells overexpressing stromelysin-1. (A) Electrophoretic analysis of total DNA (20 μg) from control (C) and IPTG-induced cells (I) after 72 hours. Corresponding RNA blot (20 μg per lane) hybridization with a probe that detects a 1.9-kb stromelysin-1 mRNA. In situ analysis of DNA from mammary gland of normal mice in midpregnancy (B) and in transgenics expressing stromelysin-1 (14) (C). Note the increase in the number of epithelial cells undergoing apoptosis and the collapsed alveoli in the transgenics compared to normal mice. Scale bar, 33 μm.

FIGURE 3



Inhibition of apoptosis in CID-9 cells. (A) Electrophoretic analysis of DNA (20 μg) from CID-9 untransfected control cells (P) or cells transfected with crmA and cultured on plastic for 7 days. (B) Quantitation of fragmented DNA from untransfected CID-9 cells (control), cells transfected with crmA, or cells treated with 0.5, 3.5, or 5.0 μM BACMK.

FIGURE 4



ICE mRNA and protein expression in mammary epithelium. (A) RNA blot (20 μg per lane) hybridized with a probe that detects a 1.6-kb ICE mRNA in mammary tissue from normal mice lactating for 9 days (L) or after involution for 2, 4, and 8 days (21, 41, 81). (B) RNA blot for ICE mRNA in CID-9 cells after 5 days of culture on ECM (E) or tissue culture plastic (P). (C) Immunoblot analysis of ICE protein in lysates from corresponding cells with a polyclonal antibody that detects the 45-kD precursor, the active 20-kD subunit, and processing intermediates (19).