Rescue of Mammary Epithelial Cell Apoptosis and Entactin Degradation by a Tissue Inhibitor of Metalloproteinases-1 Transgene

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Abstract. We have used transgenic mice overexpressing the human tissue inhibitor of metalloproteinases (TIMP)-1 gene under the control of the ubiquitous β-actin promoter/enhancer to evaluate matrix metalloproteinase (MMP) function in vivo in mammary gland growth and development. By crossing the TIMP-1 transgenic animals with mice expressing an autoactivating stromelysin-1 transgene targeted to mammary epithelial cells, we obtained a range of mice with genetically engineered proteolytic levels. The alveolar epithelial cells of mice expressing autoactivating stromelysin-1 underwent unscheduled apoptosis during late pregnancy. When stromelysin-1 transgenic mice were crossed with mice overexpressing TIMP-1, apoptosis was extinguished. Entactin (nidogen) was a specific target for stromelysin-1 in the extracellular matrix. The enhanced cleavage of basement membrane entactin to above-normal levels was directly related to the apoptosis of overlying mammary epithelial cells and paralleled the extracellular MMP activity. These results provide direct evidence for cleavage of an extracellular matrix molecule by an MMP in vivo.

THE balance between extracellular proteolytic enzymes and their inhibitors is likely to be an important modulator of cell–extracellular matrix (ECM)[1] interaction. Matrix metalloproteinases (MMPs) are usually considered to be essential to resorptive and lytic processes, and notably to the invasive lysis associated with traversing basement membranes. Indeed, an effective way to regulate the invasive behavior of normal and metastatic cells is to manipulate the concentration of one of the principal inhibitors of the MMP group of secreted proteinases, TIMP-1 (the tissue inhibitor of metalloproteinases). For example, expression of a transfected TIMP-1 gene lowers the metastatic potential of melanoma cells (Khokha, 1994), and ablation of the TIMP-1 gene results in the enhanced invasion of normal differentiated cells and altered metastasis of transformed cells (Alexander and Werb, 1992; Soloway et al., 1996). The fact that MMPs can cleave ECM proteins and facilitate invasion prompted us to ask what the effect would be of perturbing MMP expression on cells that have a clearly established ECM dependence.

Mammary epithelial cells have been shown to depend on specific ECM components for the expression of differentiated functions in vitro (Streuli, 1993; Lochter and Bissell, 1995;), making it simple to evaluate functional changes in the cell–ECM interface by measuring milk protein expression. Unexpectedly, overexpression of active stromelysin-1 in the mouse mammary gland resulted in profound changes of cell function and survival that included hyperplasia of the virgin gland and epithelial cell death in the late pregnant gland, resulting in smaller lactating alveoli (Sympson et al., 1994; Boudreau et al., 1995). These observations implicate the MMP family of secreted proteinases in growth control and morphogenesis of mammary gland. Stromelysin-1 and other MMPs are expressed in the mouse mammary gland during pregnancy; thus, the endogenous enzyme may serve in normal growth control, and this function may be usurped by expression of the transgenic autoactivating enzyme. To address this issue, we made transgenic mice overexpressing the MMP inhibitor TIMP-1 and used them to assess the normal role of MMPs. By crossing mice overexpressing TIMP-1 with mice overexpressing stromelysin-1, we were able to evaluate the bioactivity of the TIMP-1 transgene by looking for reversal of the phenotype induced by stromelysin-1. Control, stromelysin-overexpressing, and TIMP-1–overexpressing mice, together with the heterozygous cross, have a spectrum of altered proteolytic genotypes that were used to identify molecular targets for stromelysin-1.

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1. *Abbreviations used in this paper*: ECM, extracellular matrix; hTIMP-1, human TIMP-1; MMP, matrix metalloproteinase; p.c., postcoitum; S, stromelysin-1 transgenic; ST, human TIMP-1 and stromelysin-1 double transgenic; T, human TIMP-1-expressing; TIMP, tissue inhibitor of metalloproteinases; WAP, whey acidic protein.

Materials and Methods

Materials

Enhanced chemiluminescence (ECL) reagents were from Amersham (Arlington Heights, IL). Immobilon P was from Millipore Corp. (Bedford, MA). cDNA probes were human TIMP-1 (hTIMP-1) cDNA (Gasson et al., 1985), mouse TIMP-1 (Alexander and Werb, 1992), mouse keratin 18 (Kulesh and Oshima, 1988), mouse entactin (courtesy of Dr. A.E. Chung, University of Pittsburgh, PA) and mouse stromelysin-1 (Sympson et al., 1994). Sources of antibodies were as follows: antifibronectin primary antibody (rabbit polyclonal antibody to mouse fibronectin; catalog No. A117; GIBCO BRL, Gaithersburg, MD); antilaminin primary antibody (rabbit polyclonal antibody to mouse laminin; catalog No. 40023; Collaborative Research Inc., Lexington, MA); antientactin primary antibody (rat mAb to mouse entactin; catalog No. 05-208; Upstate Biotechnology, Inc., Lake Placid, NY); two other pairs of antibodies to entactin, one set of rabbit polyclonal antibodies to specific domains of entactin, namely the G1 domain and the "E" domain linking the G2 and G3 domains (courtesy of Dr. A.E. Chung, University of Pittsburgh, Pittsburgh, PA; Hsieh et al., 1994), and one set of mouse mAbs to the G1 and G3 domains (courtesy of Dr. J.W. Fox, University of Virginia, Charlottesville, VA; Reinhardt et al., 1993); antibromodeoxyuridine antibody (catalog No. 7580; Becton Dickinson & Co., Mountain View, CA); secondary antibody for rabbit primary antibodies (HRP-conjugated donkey anti–rabbit IgG; catalog No. NA9340; Amersham); secondary antibody for rat primary antibody (HRP-conjugated sheep anti–rat IgG; catalog No. NA9320; Amersham); and secondary antibody for mouse primary antibody (FITC-conjugated sheep anti–mouse IgG; catalog No. N1031; Amersham).

Assembly of the Transgene Construct

4.3 kb of the 5′ sequence from the human β-actin gene (including 3 kb of flanking sequence, 78 bp of noncoding sequence, and 832 bp of intron 1) was placed upstream of a HindIII cloning site and 877 bp of 3′ untranslated sequence from the same gene in a Bluescript vector (Stratagene, La Jolla, CA). A full-length human cDNA for TIMP-1 (an EcoRI fragment tailed with HindIII linkers) was inserted, and the whole construct was removed by cleavage with KpnI and XbaI for microinjection into the pronuclei of CD-1 mouse eggs with the use of standard transgenic technology (Hogan et al., 1994). The transgenic mouse line m2-5, which expresses autoactivating rat stromelysin-1 from the whey acidic protein (WAP) promoter, was described previously (Sympson et al., 1994).

Mammary Gland Protein Lysates

Pieces of gland tissue were homogenized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 nM Tris-HCl, pH 8.0) at 0.25 mg wet wt/μl. Lysates were spun for 15 min at 4°C in a microcentrifuge, and soluble fractions were removed for estimation of TIMP-1 inhibitor concentrations. Insoluble fractions (ECM-enriched fractions) were washed once in RIPA and boiled into SDS sample buffer.

Measurement of hTIMP-1 Concentrations

hTIMP-1 was measured in tissue lysates and serum by using a radioimmunoassay. Serum was obtained from the tail veins of homozygote and heterozygote hTIMP-1 transgenic female mice, and the concentration of hTIMP-1 was measured by standard radioimmunoassay with a rabbit polyclonal antibody raised against hTIMP-1 (which shows no cross-reaction with mouse TIMP-1) and competition with pure radioiodinated hTIMP-1 (Bullen et al., 1995). Background values, determined from control animals, were subtracted.

Analysis of Conditioned Medium by Immunoblotting and Zymography

Medium conditioned by embryonic fibroblasts isolated from 16.5-d post coitum (p.c.) embryos was prepared by overnight incubation of semiconfluent fibroblasts in serum-free medium (DME with 0.2% lactalbumin hydrolysate and 0.4% UltroSer; IBF Biotechnics, Columbia, MD). The resulting medium was diluted into sample buffer without dithiothreitol for zymography, or was boiled into reducing sample buffer for SDS-PAGE. Gelatin zymography for visualization of proteinases and reverse gelatin zymography for visualization of proteinase inhibitors were done according to the methods described by Alexander and Werb (1992). Immunologic blots of proteins transferred to Immobilon P were probed with the antibody to hTIMP-1 used for the radioimmunoassay, and were developed using the ECL system according to the manufacturer's instructions.

Histology and Immunochemistry

Mammary glands from 16-d pregnant CD-1 females of all four genotypes were fixed for 4 h in 4% fresh paraformaldehyde and embedded in paraffin for sectioning and staining with hematoxylin and eosin by standard techniques. Mitotic indices from the same series of mice were measured by injecting mice 2 h before killing with 0.1 mg bromodeoxyuridine/g body wt. Glands were infiltrated with 20% sucrose and embedded in OCT, and 10-μm sections were stained with antibromodeoxyuridine according to the manufacturer's instructions.

Results

TIMP-1 Transgenic Mice Express Inhibitor in Tissues and Serum

We made a construct consisting of hTIMP-1 and driven by a human β-actin promoter and 3′ untranslated region (Fig. 1 *a*) that would reduce the endogenous activity of MMPs and serve as a probe of their normal function in transgenic mice. mRNA for hTIMP-1 was expressed in every tissue examined at expression levels that paralleled the endogenous β-actin gene expression (data not shown). hTIMP-1 protein was present in the serum of transgenic mice at up to 50 ng/ml (Fig. 1 *b*). The serum level of hTIMP-1 heterozygote transgenic mice was about half of that in homozygotes. Fibroblasts from hTIMP-1–expressing (T) mouse embryos (Fig. 1 *c*) secreted hTIMP-1 that comigrated with mouse TIMP-1 and had inhibitory activity. Expression of MMP and other TIMP activities seen by zymography and expression of endogenous mouse TIMP-1 mRNA was unchanged by transgene expression (Fig. 1, *d* and *e*).

The elevated levels of hTIMP-1 in tissues and plasma produced no dramatic changes during embryonic mouse development (14–18 d p.c.). Although endogenous TIMP-1 shows highly regulated expression patterns in developing bone and in cycling ovaries of mice (Nomura et al., 1989), the morphology or function of most adult tissues in T mice appeared unaffected. Skeletons of transgenic embryos showed little effect of hTIMP-1 on bone morphogenesis, and there was no change in the morphology or size of calvaria and long bones of adult mice. T females were fertile (Alexander et al., 1996) and appeared to remain fertile for as long as nontransgenic siblings. Mammary glands of T mice had normal lactational capacity. A continuous excess of hTIMP-1 did not appear to alter general ECM turnover or accumulation. Using Masson's trichrome staining as a measure of collagen accumulation, we saw no generalized fibroses in a variety of tissues, even at 1 yr of age (data not shown). All in all, the transgene was remarkably benign.

Crossing TIMP-1-Overexpressing Mice with Stromelysin-1 Transgenic Mice Reverses Precocious Involution

To assess the bioactivity of the hTIMP-1 transgene, we crossed T mice with mice ectopically expressing rat stromelysin-1 directed to mammary epithelial cells by the WAP promoter (Sympson et al., 1994). These crosses generated

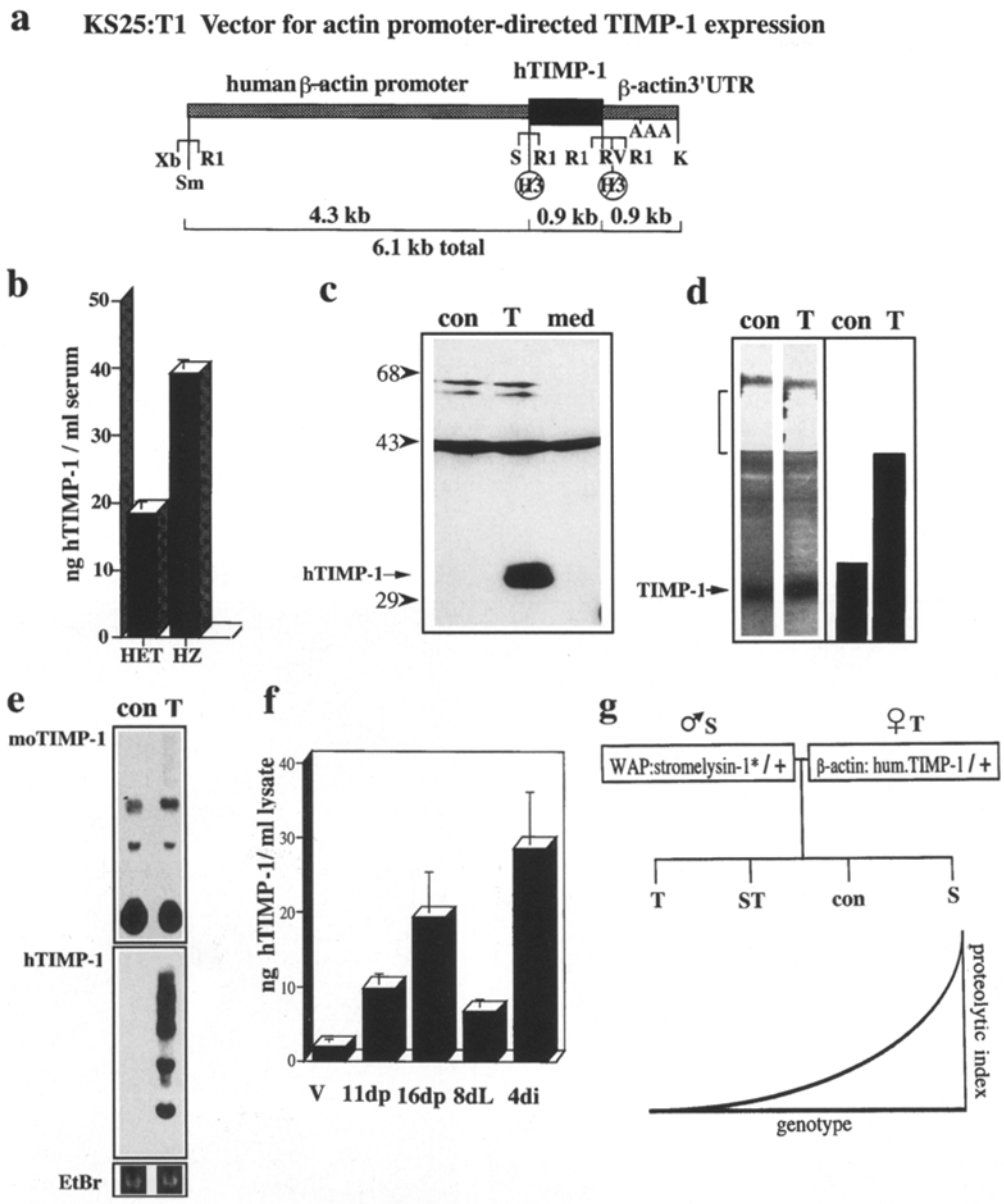

Figure 1. β-actin gene promoter–driven ubiquitous expression of transgenic hTIMP-1 in mice. (*a*) Scheme of vector construct. 4.3 kb of the human β-actin promoter drives expression of a hTIMP-1 cDNA, with the β-actin 3′ untranslated region (*UTR*) and poly A site (*AAA*). The restriction sites shown are XbaI (*Xb*), SmaI (*Sm*), EcoRI (*R1*), SalI (*S*), EcoRV (*RV*), and KpnI (*K*). Two HindIII (*H3*) sites were deleted during construction of the vector. (*b*) Circulating levels of hTIMP-1 in heterozygote (*HET*) and homozygote (*HZ*) transgenic mice. Bars indicate standard error. (*c*) Expression of transgenic TIMP-1 by cultured cells. Proteins secreted into serum-free medium by embryonic fibroblasts (equivalent of proteins secreted by 10^5 cells/20 h per lane) derived from heterozygote T (*T*) and control wild-type (*con*) 16.5-d p.c. sibling mouse embryos were separated by SDS-PAGE, blotted to Immobilon P, and probed with anti–hTIMP-1 antibody. The third lane (*med*) contains serum-free tissue culture medium. Molecular weight standards (in kilodaltons) are shown at left. (*d*) Inhibitor activity of transgenic protein. Conditioned medium from the experiment described in *c* was analyzed by reverse zymography (*con*, wild-type control fibroblasts; *T*, transgenic fibroblasts). Gelatinolytic activities are revealed as white bands (indicated by bracket on left margin), and inhibitors of gelatinolytic activities are revealed as dark bands. Cultured cells typically express endogenous TIMP-1; in this case, synthesis of the 29-kD TIMP-1 inhibitor was effectively doubled by expression of the transgenic construct (*TIMP-1*). The scanning densitometric evaluation of inhibitory activity is shown in the panel on the right. The values are normalized to control gland as 1.0. (*e*) mRNA expression of the endogenous and transgenic TIMP-1 genes in embryonic fibroblasts. mRNA was prepared from embryonic fibroblasts from control wild-type (*con*) and transgenic (*T*) embryonic fibroblasts and RNA blots with 10 μg of RNA per lane, and were probed with either a mouse (*moTIMP-1*) or human (*hTIMP-1*) TIMP-1 cDNA probe. (*EtBr*, ethidium bromide–stained 18S rRNA bands.) (*f*) Expression of the TIMP-1 transgenic protein during mammary gland development. hTIMP-1 was measured in the RIPA-soluble fraction of mammary gland lysates by radioimmunoassay. *V*, virgin; *dp*, days pregnant; *dL*, days of lactation; *di*, days of involution. (*g*) Cartoon of the strategy used to generate an array of proteolytic genotypes in the mammary gland. Heterozygous male mice carrying the stromelysin transgene, directed by the WAP promoter (*S*), were crossed with heterozygous females carrying the human TIMP-1 transgene, directed by the β-actin promoter (*T*). Their offspring segregated into four different genotypes, carrying either transgene alone (*T* and *S*), none at all (*con*), or both (*ST*). These genotypes are arrayed in increasing order of amount of extracellular proteolysis. Female pups at maturity were mated, and timed pregnant glands were used for the analyses.

four types of animals with distinct proteolytic genotypes: wild-type (control), T, stromelysin-1 transgenics (S), and double heterozygotes carrying both hTIMP-1 and stromelysin-1 transgenes (ST). In mammary gland lysates from T females, hTIMP-1 protein increased in concentration throughout pregnancy (Fig. 1 *f*) and then decreased in lactating glands (in parallel with expression of the endogenous actin gene), accumulating again after weaning.

Transgenic stromelysin-1 expression in the S animals produced partial unscheduled involution of the late pregnant mammary gland by inducing basement membrane degradation and apoptosis of mammary epithelial cells.

This led to the significantly smaller alveolar cross section characteristic of S glands during lactation (Fig. 2). By in situ analysis, S glands were indistinguishable from controls at 10.5 d p.c., but had approximately sevenfold increased numbers of apoptotic cells at 16.5 d p.c. compared to control (data not shown). Coexpression of the TIMP-1 transgene with stromelysin-1 in lactating glands of ST animals restored the alveolar volume to control size, as determined by histologic evaluation of stained sections (Fig. 2). To confirm that this rescue was the result of suppression of proteinase-induced apoptosis, we evaluated cell growth and apoptotic rates in late pregnant (16.5 d p.c.) glands. To assess the proliferative rates in all four types of glands, we injected mice with bromodeoxyuridine, removed the glands 90 min later, and stained the sections with an antibody to bromodeoxyuridine. All four types of glands showed similar proliferative rates (Fig. 2 *c*). However, the apoptotic cell death seen in the stromelysin-1 transgenics by means of analysis of DNA extracts for nuclease-dependent laddering was effectively reversed in the ST double transgenics (Fig. 3). Intriguingly, even in the presence of elevated expression of the stromelysin-1 transgene, MMP-dependent apoptosis ceased during lactation (Fig. 4), when epithelial proliferation ceased (data not shown).

Upregulation of Endogenous Stromelysin-1 in Stromelysin-1 Transgenic Mice Is Reversed by Overexpression of the TIMP-1 Transgene

Stromelysin-1 is upregulated during normal postlactational mammary gland involution (Talhouk et al., 1992; Lund et al., 1996). We noted that ectopic expression of rat stromelysin-1 in 16.5-d p.c. glands induced the expression of endogenous mouse stromelysin-1 mRNA (Fig. 4) when

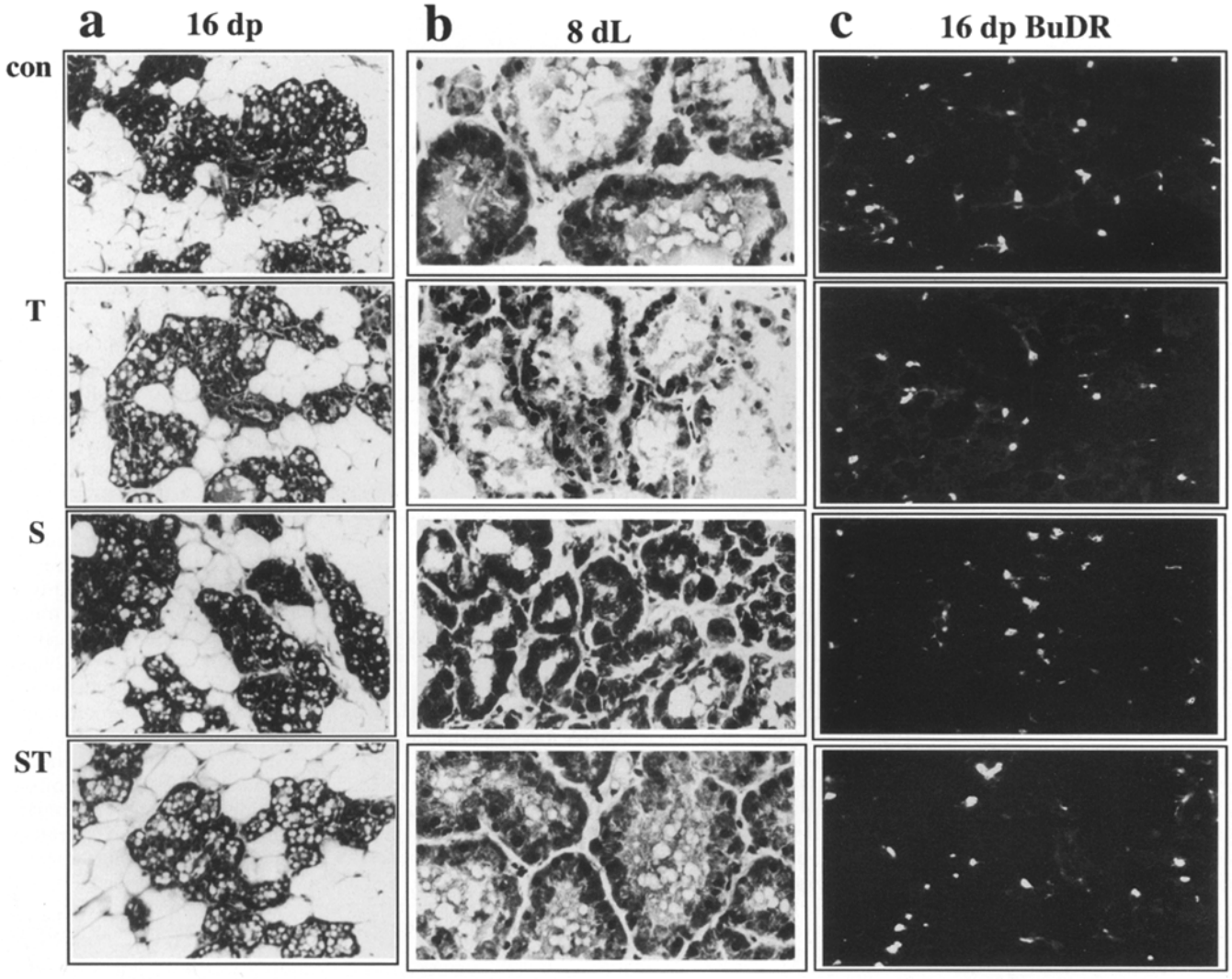

Figure 2. Morphologic evaluation of mammary glands with four different genotypes. 16.5-d p.c. glands from control (*con*), TIMP-1–expressing (*T*), and stromelysin-1–expressing (*S*) mice and from mice carrying both genes (*ST*) were evaluated by (*a*) Masson's trichrome staining of paraformaldehyde-fixed paraffin sections (*16 dp*) and showed no genotype-specific phenotype. (*b*) Frozen sections of 8-d lactating glands (*8 dL*) showed that although the S mammary glands had the characteristically reduced alveolar volume, this phenotype was restored to normal by the coexpression of the TIMP-1 transgene (*ST*). T glands were indistinguishable from controls. (*c*) The mitotic index of these glands was similar in all four types, as measured by staining glands from mice injected with bromodeoxyuridine (*BuDR*) with an FITC-conjugated anti-BuDR antibody.

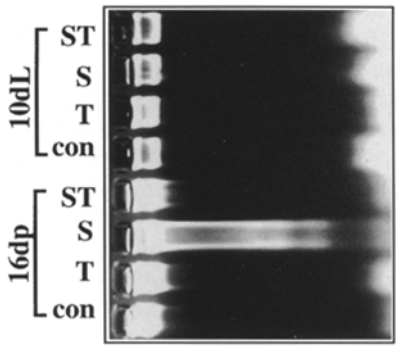

Figure 3. Analysis of the rescue of stromelysin-1–dependent apoptosis by expression of the TIMP-1 transgene. Analysis of cell death in mammary glands of all four genotypes. DNA was extracted from pregnant and lactating mammary glands of all four genotypes (see Fig. 2) and analyzed for apoptotic laddering on 2% agarose gels (Boudreau et al., 1995). Samples from 16-d p.c. pregnant glands (*16dp*) show that the apoptotic laddering induced by the stromelysin-1 transgene was reversed by the coexpression of the TIMP-1 transgene (*ST*). During lactation (*10dL*), the WAP promoter increased transgenic expression of stromelysin-1; however, the mammary epithelial cells became resistant to apoptosis during this developmental phase.

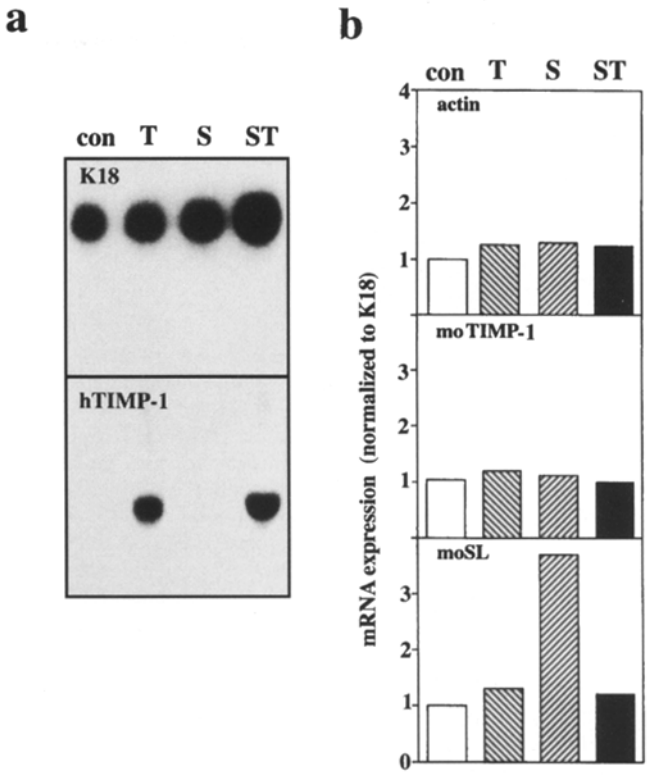

Figure 4. RNA blot analysis of transgene expression and reversal of stromelysin-1–induced amplification of endogenous gene expression by exogenous TIMP-1. 10 μg of RNA extracted from 16.5-d p.c. mammary glands of control (*con*), T, S, and ST mice (defined in the legend to Fig. 2), was analyzed for the expression of several mRNA species: (*a*) Keratin 18 (*K18*, an epithelial cell marker, used here as an internal standard; *top*) and hTIMP-1 transgene (*bottom*). (*b*) Scanning densitometric quantification of RNA blots probed with actin, endogenous mouse TIMP-1 (*moTIMP-1*), or mouse stromelysin-1 (*moSL*) normalized with respect to the internal keratin 18 (*K18*) standard, where control (*con*) values are set at 1.0. There were no significant differences in the expression of endogenous mouse TIMP-1 in the presence of transgenic TIMP-1 mRNA. The three- to fourfold upregulation of endogenous stromelysin-1 expression observed with the S transgene was effectively reversed by the coexpression of hTIMP-1 mRNA.

precocious involution was extensive. The rat stromelysin-1 transgene was expressed at very low levels, detectable only by reverse transcription followed by PCR, and did not contribute to the signal seen by RNA blotting (Sympson et al., 1994). Accordingly, we checked to see if this secondary molecular phenotype of involution in stromelysin-1 transgenic mice was reversed in ST mice. There was no significant alteration in actin/keratin 18 mRNA ratio in any type of gland (Fig. 4, *a* and *b*), confirming morphologic observations that the epithelial/stromal cell ratio was unchanged. Expression of hTIMP-1 mRNA was identical in T and ST animals (Fig. 4 *a*), and expression of the endogenous mouse TIMP-1 gene was not affected by expression of the transgene (Fig. 4 *b*). Interestingly, endogenous stromelysin-1 mRNA expression in T glands was similar to that in controls, but the three- to fourfold upregulation of stromelysin-1 expression typical of S glands was restored to normal levels in ST animals (Fig. 4 *b*).

We next determined whether the expression of other MMPs was affected in mice of all four proteinase genotypes. To assess the impact of the stromelysin-1/TIMP-1 balance on the activation status of MMPs, we analyzed lysates of mammary glands by zymography (Fig. 5). Gelatinase A (72 kD) was the most prominent activity in mammary gland, and its expression and activation (indicated by a decrease in molecular mass of the activity to 60 kD) were almost constant throughout pregnancy; activation ceased during lactation. Because gelatinase A expression and activation were at the same low levels in all four types of glands at 16.5 d of pregnancy, we concluded that neither stromelysin-1 nor TIMP-1 expression affects gelatinase A activation in transgenic mice. This suggests that (*a*) the autoactivating stromelysin-1 is not initiating a cascade of MMP activation, as is the case with purified enzymes in vitro (Sorsa et al., 1992); and (*b*) gelatinase A is not directly involved in the induction of apoptosis.

Entactin Is a Molecular Target for Proteolysis during Normal and Unscheduled Apoptosis

Next, we exploited the four proteolytic genotypes to look for molecular targets in the ECM that underlie the morphologic changes. In particular, we searched for a stromelysin-1–dependent physiologic effector of the apoptotic response during late pregnancy. Protein extracts enriched for ECM were separated on 6% SDS-polyacrylamide gels and showed no gross changes. A representative profile of proteins extracted by SDS-PAGE sample buffer from ECM-enriched fractions of mammary glands is shown in Fig. 6 *a*. This profile was dominated by collagens, and these proteins showed some quantitative variability between the samples that was genotype independent. We next examined the profiles for specific ECM proteins by immunoblotting, followed by quantification by means of scanning densitometry. Interestingly, when normalized for

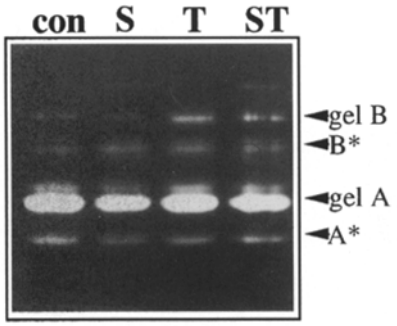

Figure 5. Analysis of MMP expression in mammary glands of different proteolytic genotypes. RIPA lysate supernatants (described in the legend to Fig. 1 *f*) of glands from control (*con*), S, T, and ST mice were analyzed by gelatin zymography (described in the legend to Fig. 1 *d*) on 10% polyacrylamide gels. Gelatinase A (72-kD gelatinase; *gel A*) predominates in the profile, and the amount of the activated cleavage product at 60 kD (*A**) is independent of proteolytic genotype. Gelatinase B (92-kD gelatinase; *gel B*) and its activated cleavage product (*B**) are minor constituents, but show the same independence of genetic background.

total extractable protein, laminin, which is bioactive in determining the differentiated function of mammary epithelial cells in vitro (Streuli et al., 1995), and fibronectin were present in similar amounts, and there were no prominent smaller bands that would imply accumulation of MMP-specific cleavage products in these late pregnant glands (Fig. 6, *b* and *c*). However, entactin (nidogen), the major basement membrane cross-linker between laminin and collagen, showed significant changes (Fig. 6, *d–f*). Although the total amount of entactin mRNA (data not shown), the amount of extractable entactin protein relative to laminin (Fig. 6 *f*), and the solubility characteristics of this protein were not significantly affected by the level of extracellular proteolysis, we identified two major cleavage products (110 and 100 kD) of entactin accumulating in extracts of pregnant glands. These fragments have also been observed in lysates of the developing salivary gland (Kadoya et al., 1995) and in Engelbreth-Holm-Swarm Sarcoma matrix preparations (Ekblom et al., 1994). These cleavage products paralleled the proteolytic genotype of the gland (Fig. 6 *d*). Control glands contained low amounts of these two cleavage products (~4% of total entactin), whereas S glands contained elevated levels, especially the 110-kD fragment (up to 40% of total entactin protein). This is in keeping with observed low but significant numbers of apoptotic cells by in situ analysis in the control glands (data not shown). Raising the local MMP inhibitor concentration in the T and ST animals resulted in greatly reduced amounts of these proteolytic fragments to levels even lower than control (Fig. 6, *d* and *e*).

If entactin degradation is related to mammary gland apoptosis, then its fragments should also be evident during the normal epithelial involution induced by postlactational weaning. Half of the extractable entactin appeared in the 110- and 100-kD fragments in control animals weaned 4 d after 8 d of lactation (Fig. 6, *d* and *e*).

Within the tertiary structure of entactin, there is a domain on the NH_2-terminal flank of the G2 domain that is selectively cleaved by proteinases of several classes (Mayer et al., 1993) and also contains an epitope that confers β_1-integrin binding (Fig. 7). To confirm that the 110- and 100-kD proteins also reacted with other antientactin antibodies in these crude ECM-enriched lysates, and to identify the site of cleavage in the gland in vivo, we probed Western blots of involuting gland lysates with two sets of domain-specific antibodies, one polyclonal and the other monoclonal. Antibodies to the rod domain separating G2 and G3 and antibodies to G3 reacted with all three proteins (150, 110, and 100 kD), whereas antibodies to G1 reacted only with the native 150-kD protein. From the size of the fragments and their lack of G1 epitopes, we conclude that approximately the same site is cleaved in vivo as in vitro. The collagen IV–binding G2 domain and the laminin-binding G3 domain remain linked, maintaining the interconnecting bridge, and the G1 domain (of unknown function) is clipped off.

Discussion

The significance of these observations is threefold: (*a*) Systemic overexpression of TIMP-1 does not produce major morphologic changes in the mouse or, more particularly, during mammary gland development in late pregnancy and lactation. This conclusion is derived from simple histologic evaluation. (*b*) Transgenic expression of an active MMP results in the proteolytic cleavage of a target ECM molecule (entactin) in vivo; and (*c*) the biologic properties of entactin imply that this cleavage may have an effector role in inducing the apoptotic phenotype.

Although the MMPs can cleave various components of the ECM in vitro (Birkedal-Hansen et al., 1993), there has been little direct evidence for physiologic MMP-dependent proteolysis of ECM molecules. A neoepitope of aggrecan that is likely to be generated by MMP proteolysis has been immunolocalized in articular cartilage during inflammatory arthritis (Singer et al., 1995). Fragments of link protein-3 that have been isolated from young cartilage show the same cleavage specificity as those produced in vitro by incubation with purified stromelysin-1 (Hughes et al., 1992). Purified entactin is notably susceptible to proteolytic cleavage by various classes of proteinase (Mayer et al., 1993; Sires et al., 1993), including the MMPs stromelysin-1 and matrilysin. Entactin is a 150-kD extended molecule, comprising three globular domains linked by a flexible sequence and a rigid rod (E domain; Fig. 7). The second globular domain (G2) has an affinity for collagen type IV, and the third globular domain (G3) binds laminin (Aumailley et al., 1993; Chung et al., 1993). The function of the first globular domain (G1) is so far unknown. Proteinases cleave entactin according to their own specificity, but usually within the flexible linker that connects the G1 and G2 domains. This cleavage typically generates fragments of 110 and 100 kD (Mayer et al., 1993).

Entactin is a mediator of mesenchymal–epithelial crosstalk; it is produced by mesenchymal cells and deposited in the subepithelial basement membrane (Warburton et al., 1984; Thomas and Dziadek, 1993; Ekblom et al., 1994). Entactin accelerates the formation of macromolecular clusters of laminin and collagen type IV in vitro, implying a bridging or structural role in the assembly of basement membranes. The proteolytic susceptibility of entactin after

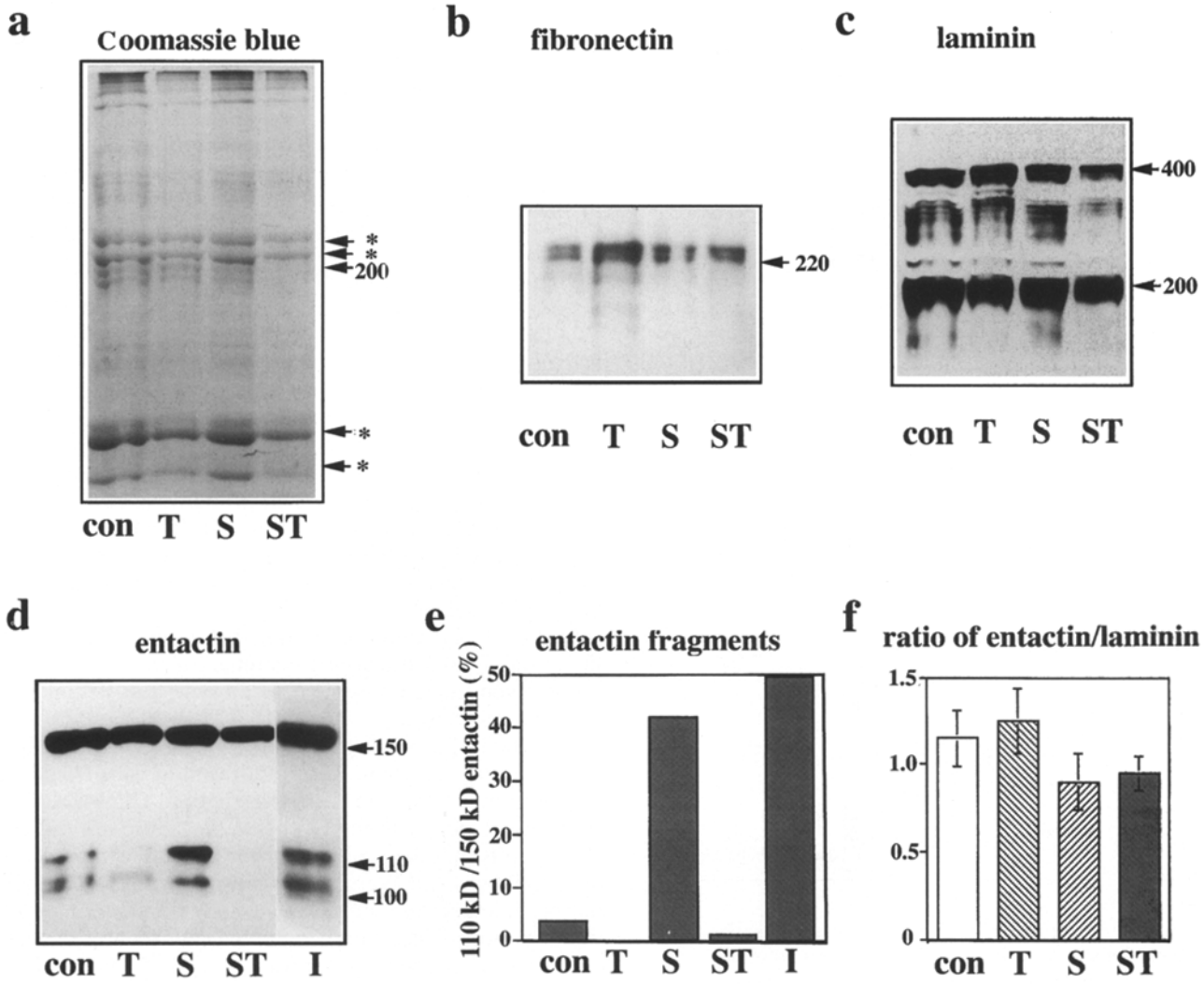

Figure 6. Biochemical analysis of ECM fractions from 16.5-d p.c. mammary glands with four different proteolytic genotypes (*con*, *T*, *S*, and *ST*, as defined in the legend to Fig. 2) and from RIPA-insoluble lysates prepared from involuting mammary glands from control mice that were allowed to lactate for 8 d and were weaned for 4 d (*I*). (*a*) RIPA-insoluble fractions were boiled for 3 min in sample buffer containing 5% SDS, and extracted proteins were separated on 6% SDS-polyacrylamide gels. The gel was stained with Coomassie blue to evaluate the total protein pattern in control (*con*), T, S, and ST glands. Bands indicated with an asterisk (*) were sensitive to digestion with clostridial collagenase. (*b–f*) Evaluation of specific ECM components in ECM-enriched fractions from all four types of glands. Replicate gels of the extracts shown in *a* were blotted to Immobilon P and probed with specific antibodies to ECM components: (*b*) antifibronectin; (*c*) antilaminin; and (*d*) antientactin. (*e*) Quantification of the 110-kD proteolytic fragment of entactin in the four types of pregnant gland and normal involuting gland. Quantitative data from the gel shown in *d*, a representative example of entactin profiles from the four types of pregnant gland and normal involuting gland, were obtained from four separate blots by means of scanning densitometry. (*f*) Quantification of ratios of entactin and laminin in the four types of gland. Scanning densitometry of bands on immunologic blots from several extracts, analyzed as described for *c* and *d*, was used to quantify the relative amounts of entactin and laminin in each genotype. The ratio of entactin to laminin was not genotype dependent. Bars indicate standard error.

assembly into basement membrane networks is unclear, although when it forms a complex with the laminin P1 domain, the secondary cleavage site that generates the 100-kD fragment in vitro is protected (Mayer et al., 1993). In addition to its structural properties, entactin binds to cell-surface receptors, specifically the $\alpha_3\beta_1$-integrin (G2 domain) (Dedhar et al., 1992) and the $\alpha_V\beta_3$-integrin (E domain). In pure form, entactin can alter attachment and migratory reactions in a number of cell types in culture (Chung et al., 1993; Sires et al., 1993). Function-perturbing antibodies to the entactin/nidogen binding site of the laminin-γ1 chain inhibit growth and branching morphogenesis of lung and kidney epithelial cells in vitro (Ekblom et al., 1994). The accessibility of the integrin-binding sites of entactin enmeshed in basement membranes is unknown. Both the structural and cell-surface ligation properties of entactin are suggestive of effector roles that could be modulated by proteolysis. First, raising the quantity of cleaved entactin from 4 to 40% of the total could lower the integrity of the basement membrane enough to induce cell death in a number of the overlying cells; or second, cleaved entactin could generate an integrin-mediated signal that leads to

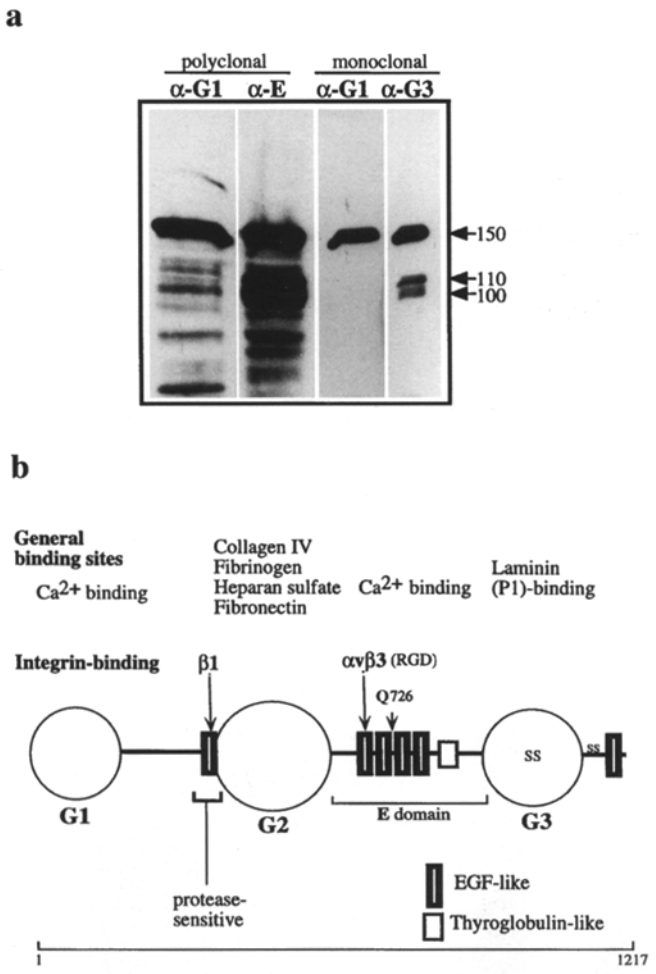

Figure 7. Determination of MMP-dependent cleavage site in entactin in mammary gland in vivo. ECM extracts from involuting glands of CD-1 females (weaned 4 d after 8 d of lactation) were separated as described in Fig. 6 and probed in Western blots with two pairs of epitope-specific antibodies to entactin. (*a*) Polyclonal rabbit antibodies to the E and G1 domains and monoclonal mouse antibodies to the G1 and G3 domains showed the same pattern of reaction. All four antibodies reacted with native entactin (150 kD), but the anti-G1 antibodies did not react with the 100/110–kD derivative proteins. Removal of a 40–50-kD fragment containing all the G1 epitopes by MMP-dependent cleavage is consistent with the site identified in vitro as a proteinase-sensitive zone. Relevant domains and functions in the entactin molecule are cartooned in *b*. The proteinase-sensitive and integrin-binding sites are marked, together with the binding affinities of each specific domain.

apoptosis. In a study published in 1980, the administration of *cis*-hydroxyproline (which inhibits the secretion and production of collagens, including collagen IV) induced apoptosis in mammary epithelial cells dividing in response to steroid injection (Wicha et al., 1980). Histologic evaluation of basement membranes from treated glands showed they were disorganized and fragmented, leading the authors to conclude that this was the cause of epithelial cell death. ECM components interact to stabilize one another and to stabilize their cognate cell surface receptors (Wu et al., 1995); therefore, changes in one component are likely to affect several others. The issue under investigation here is what constitutes a basement membrane that sustains the overlying cells: is it a level of structural integrity, or a series of specific ECM–cell interactions?

Modulation of cell surface integrin–ECM interaction by MMP activity is not without precedent. Our earlier studies showed that fibronectin or collagen substrates, stromelysin-1 expression, and function-perturbing β_1-integrin antibodies all induce apoptosis of mouse mammary epithelial cells in culture (Boudreau et al., 1995). Indeed, proteolytic fragments of a complex basement membrane not only fail to support differentiation of mammary epithelium in vitro, but also act in a dominant-negative manner to inhibit ECM-mediated differentiation (Streuli et al., 1995). MMP activity expressed by melanoma cells plated on a collagen gel substrate in vitro changes the basis of the cell–ECM interaction from ligation of the $\alpha_2\beta_1$-integrin to an α_v-mediated interaction (Montgomery et al., 1994). Clearly, different intracellular signals are generated by the ligation of different integrin receptors with proteolytic fragments of ECM molecules (Huhtala et al., 1995). It will be interesting to test the possibility that entactin is an intermediary for stromelysin-1–dependent apoptosis of cultured mammary epithelial cells.

Our surprising observation was that apoptosis ceased in the stromelysin-1 transgenic mammary glands during lactation, even though basement membrane destruction was maximal (Sympson et al., 1994). Cell proliferation was equally high during pregnancy in all four genotypes, but ceased during lactation. The withdrawal of cells from the cell cycle may eliminate their sensitivity to ECM-directed apoptosis. Upon entering the cell cycle, there are critical checkpoints that determine whether the cell will divide or die (Ashkenas and Werb, 1996). In support of this model, our preliminary data indicate that during involution, after weaning, normal mammary epithelial cells reenter the cell cycle before apoptosis (MacAuley, A., L. Lund, and Z. Werb, unpublished observations). Moreover, we have observed that mammary epithelial cells in culture enter the cell cycle before initiating apoptosis (Boudreau et al., 1996). Pregnant, lactating, and involuting mammary glands of all four genotypes should provide a novel system in which to evaluate the contribution of cell cycle to apoptosis in vivo.

Increased extracellular proteolysis is a feature of many inflammatory and degenerative diseases, and also of tumorigenesis (Sympson et al., 1995), and is implicated in ECM modification, growth factor availability, and angiogenesis (Mignatti and Rifkin, 1993). The key to understanding the role of proteinases in these processes lies in the identification of their substrates. Our biochemical evaluation of ECM targets for the stromelysin-1 transgene during stromelysin-1–induced apoptosis in the mammary gland provides a conceptual basis for the design of strategies that use proteinase inhibitors as a probe of proteinase function in disease processes. Such an approach will be useful for evaluating the functions of other members of the MMP and TIMP families.

We thank Albert Chung for reagents he gave us before publication and for many helpful discussions, Dr. J.W. Fox for gifts of antibodies, E.C. Bullen for expert technical assistance, K.S. Sturm for the development of the β-actin expression vector, E.J. Hansell for helping with the figures, and M. McKenney for editing the manuscript.

C.M. Alexander is the recipient of an Arthritis Investigator Award from the Arthritis Foundation. This work was supported by grants from the National Cancer Institute (CA 57621) and the Women's Health Initiative, Office of the Director of National Institutes of Health (CA 57621S1), and a contract from the Office of Health and Environmental Research, U.S. Department of Energy (DE-AC03-76-SF01012).

Received for publication 14 March 1996 and in revised form 15 August 1996.

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