Biochemical Changes during Growth and Regression of Pregnancy-Dependent Mammary Tumors of GR/A Mice (43906)

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Abstract. **The weights of pregnancy-dependent mammary tumors (PDMT) of GR/A mice continued to increase until parturition and decreased soon after delivery; however, mitotic indices in epithelial cells and stromal cells of PDMT reached a maximum plateau on Day 18–19 of pregnancy and decreased thereafter. Growth of PDMT in progesterone-treated mice on Day 15 of pregnancy was higher than that in 17β-estradiol-treated mice and no treatment controls. DNA fragmentation was observed in PDMT on Day 20 of pregnancy and just after parturition. Two-dimensional gel electrophoresis of PDMT extracts revealed that five and six protein spots appeared newly on Day 20 of pregnancy and just after parturition, respectively. N-terminal amino acid sequences of two of the protein spots were identical to that of α-lactalbumin. PDMT on Day 15 and 20 of pregnancy and just after parturition secreted matrilysin, one of the matrix metalloproteinases, which was identified by Western blotting. However, matrilysin was not found in hormone-independent autonomous mammary tumors of the mouse. Estrogen receptor and c-*fos* mRNA expression levels in PDMT were high on Day 15 of pregnancy but low on Day 20 of pregnancy and just after parturition. These findings suggest that regression of PDMT is caused by apoptosis, and new proteins expressed on Day 20 may participate in the process of regression.** [P.S.E.B.M. 1995, Vol 209]

The GR/A mouse is characterized by the development of pregnancy-dependent mammary tumors (PDMT), which appear at the middle of pregnancy and continue to grow until the end of pregnancy. PDMT regress or disappear soon after parturition regardless of the onset of lactation, and appear again at subsequent pregnancies. The incidence and the size of the PDMT often increase with each additional pregnancy, and PDMT often transform into hormone-independent autonomous mammary tumors (MT) (1–3).

PDMT have estrogen and progesterone receptors (4–6), and progesterone alone or in combination with estrogen stimulates DNA synthesis in PDMT cells *in vivo* (5, 7–9). PDMT appear during pregnancy when levels of estrogen and progesterone in the circulation are high (10, 11), suggesting that these hormones stimulate the growth of PDMT. Estrogen and progesterone are known to be important hormones for mouse mammary tumorigenesis, including the induction of hormone-dependent mammary tumors in virgin GR mice (2, 4). There are many reports on the growth of PDMT in GR mice (2–9); however, the regression of PDMT has not yet been studied well except for the report that tumor necrosis factor-α somewhat inhibited the growth of PDMT (12).

With regard to the regression in the prostate, it has been reported that proteins are induced during castration-induced regression in the rat. These proteins may play important roles in the regression process (13–16). In estrogen-regulated growth and regression of cells, secretion of procathepsin D, cathepsin D, and transforming growth factor-α and -β (TGFα, -β) has been

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reported (17–20). Although procathepsin and TGFα stimulate growth of breast cancer cells and uterine cells *in vitro* (18, 20), cathepsin D and TGF-β inhibit the growth (19, 21). Polypeptide growth inhibitors of the mammary gland and breast carcinomas have been purified; mammary-derived growth inhibitor and mammastatin (22–25). Among the other proteins considered to be related to growth and regression processes are matrilysin, gelatinases, and stromelysin (26–32). Stromelysin-3 mRNA is expressed in fibroblastic cells of the mammary gland undergoing apoptosis after weaning. It is also expressed in fibroblastic cells of breast carcinomas (32). Thus, stromelysin-3 is implicated in the extracellular matrix remodeling processes of mammary apoptosis and breast cancer progression.

To elucidate the mechanisms of regression and growth of PDMT, we examined the growth rate of PDMT after progesterone or E_2 treatment, the mitotic indices in epithelial and stromal cells of PDMT, the levels of expression of proteins, matrix metalloproteinases, proto-oncogenes, and estrogen receptor mRNAs, and the degree of DNA fragmentation in growing and regressing PDMT and pregnancy-independent MT in GR mice.

Materials and Methods

Animals and Reagents. GR/A Mei mice maintained by strict brother-sister mating were obtained from the Experimental Animal Laboratory, Meiji University, Kawasaki, Japan. Mice were kept under a 12:12-hr light:dark cycle at 22–24°C. Standard laboratory feed (CE-2, CLEA, Tokyo) and tap water were given *ad libitum*. At 60–70 days of age, female mice were mated with a male. Pregnant or lactating mice with palpable PDMT were sacrificed by decapitation under light ether anesthesia on Day 14–20 of pregnancy or on postpartum Day 0 (the day of parturition). At autopsy, PDMT were immediately removed and stored at −80°C for later extraction of protein, DNA, and RNA, or fixed in Bouin's solution. MT samples were collected from 6- to 7-month-old mice. Normal inguinal mammary glands (MG) with or without PDMT were also collected. Materials unless otherwise mentioned were purchased from Wako Pure Chemical, Osaka, Japan.

Histology. MG, PDMT, and MT were fixed in Bouin's solution, embedded in paraffin and serially sectioned at 8 μm. The sections were stained with Delafield's hematoxylin and eosin. The mitotic indices in MG, PDMT, and MT cells were estimated by counting the number of cells at metaphase per 500 epithelial and stromal cells of PDMT and MT in five randomly selected sections. The areas occupied by epithelial and stromal cells in a unit area (1 mm^2) of PDMT were measured by a Color Image Analyzer (Olympus, Tokyo, Japan).

DNA Fragmentation. Genomic DNA from PDMT on Day 15–20 of pregnancy, on postpartum Day 0 and from MT was prepared for examination of DNA fragmentation, which is considered to be an index of apoptosis. PDMT and MT frozen in liquid nitrogen were crushed to a fine powder in liquid nitrogen and then incubated in a digestion buffer, 100 m*M* NaCl, 10 m*M* Tris-HCl (pH 8), 25 m*M* disodium ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS) and 0.1 mg/ml proteinase K at 37°C for 12 hr. Concentration and purity of DNA were determined by measuring the optical density at 260 and 280 nm. Ten micrograms of DNA were subjected to electrophoresis on a 1.5% agarose gel and then stained with ethidium bromide.

Protein Extraction. Proteins from MG, PDMT, and MT were prepared for isoelectric focusing and polyacrylamide gel electrophoresis by a modification of the method of Wilson *et al.* (33). Fifty milligrams of tissue were vortexed without homogenization in 210 μl 8 *M* urea containing 1% SDS and 2.5% dithiothreitol (DTT; Sigma Chemical Co., St. Louis, MO) for 10 min, then vortexed again for 6 min after addition of 58 mg urea and 73.5 μl 10% NP-40 containing 0.4% ampholine (pI 3.5–10; Pharmacia LKB, Uppsala, Sweden) and 1.6% Bio-Lyte (pI 5–8; Bio-Rad, Richmond, CA). The extracts were obtained by centrifugation at 9000*g* for 1 hr. All procedures were carried out at 4°C. Protein concentration in the supernatant was determined by the method of Smith *et al.* (34) as modified by Hill and Straka (35) using BCA protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Separation of Epithelial Cells and Stromal Cells of PDMT. Epithelial cells and stromal cells of PDMT were dissociated by collagenase digestion and separated by Percoll (Pharmacia LKB) density gradient as previously reported (36). Proteins were then extracted separately as described above.

Two-Dimensional Gel Electrophoresis. Two-dimensional polyacrylamide gel electrophoresis was performed according to the method described by O'Farrell (37). Isoelectric focusing of samples (75 μg protein per gel) was performed in tube gels (2.5 mm in diameter, 13 cm in length) consisting of 1.6% Bio-Lyte (pI 5–8) and 0.4% ampholine (pI 3.5–10). The pI gradient of the isoelectric focusing gel was measured on 5-mm gel sections. The second dimension electrophoresis was conducted on 13-cm long, 2-mm thick slab gels composed of 13% polyacrylamide to resolve relatively low molecular weight proteins. Following electrophoresis, the gels were analyzed by silver staining (38, 39). The actin spot identified by Western blot analysis was used as a standard. The gels were scanned by computerized densitometry (ACI Japan, Yamato, Kanagawa, Japan) and relative O.D. was calculated

based on actin O.D. = 100. Duplicate samples from each of three separate experiments were analyzed. Only the changes consistently observed in the three separate experiments were tabulated. Intensity of actin spots was more or less the same. Thus, more protein is present in spots showing stronger intensity.

Hormone Treatment. Female mice bearing PDMT (*ca.* 4 mm in diameter) on Day 15 of the second pregnancy were given three progesterone (Sigma) pellets (each 3 mg) or one 17β-estradiol (E_2; Sigma) pellet (3 mg; E_2:cholesterol = 1:1000) as reported previously (4). Cholesterol pellets of the same weight were given to control mice. Each mouse was checked daily for palpable PDMT from Day 15 of pregnancy until disappearance of PDMT. The size of PDMT expressed in terms of the geometric mean of the major two diameters was recorded. Growing (Day 3 after parturition) and regressing (Day 6–7 after parturition) PDMT in progesterone-treated mice were dissected out and stored at −80°C for protein extraction. Regressing PDMT on Day 1 after removal of progesterone pellets were also dissected and stored at −80°C. Extracted protein samples were electrophoresed on 13% polyacrylamide gels as described above.

Amino Acid Sequence Analysis. The N-terminal amino acid sequences were determined using PSQ-1 automatic gas-phase amino acid sequencer (Simadzu, Kyoto, Japan). The proteins newly expressed on Day 20 of pregnancy in PDMT (nos. 15 and 24), and just after parturition in PDMT (nos. 20, 22, and 26) were transferred to polyvinylidene difluoride (PVDF; Immobilon-P; Millipore, Bedford, MA) membranes. The sample was placed in the reaction chamber of the amino acid sequencer, and amino acid sequencing was done by Edman degradation in an automatic mode as recommended by the manufacturer. Protein sequences were analyzed with a PC/GENE computer software (Release 6.01, IntelliGenetics Inc., Mountain View, CA).

RNA Purification and Northern Blot Analysis. Probes for v-*jun*, v-*fos* and v-*myc* (supplied by Japanese Cancer Research Resources Bank-Gene, Tokyo, Japan) estrogen receptor (supplied by Dr. K. Kano, University of Tokyo), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; supplied by American Type Culture Collection, MD) were prepared by digestion of plasmids with appropriate restriction endonucleases. The insert DNA was separated by agarose gel electrophoresis and finally purified by Ultrafree-C3 (0.45 μm, low binding durapore membrane; Millipore).

Total RNA was extracted from PDMT on Day 14, 15, 17, 19, and 20 of pregnancy and on the day of parturition, and from MT and MG on Day 14 of pregnancy by the acid guanidinium thiocyanate-phenol-chloroform extraction method (40). Concentration and purity of total RNA were determined by measuring the optical density at 260 and 280 nm. Ten micrograms of RNA from each sample were denatured and electrophoresed on 2.2 *M* formaldehyde-1.2% agarose gels, then stained with ethidium bromide to assess the quality of RNA by the presence of two sharp bands for 28 S and 18 S ribosomal RNA. After electrophoresis, RNA was transferred to a nylon membrane (Hybond-N; Amersham, Chicago, IL) overnight using the standard capillary blotting techniques in 20 X SSPE (3.6 *M* NaCl, 0.2 *M* dibasic sodium phosphate, 20 m*M* EDTA). RNA was then cross-linked to the membrane by UV irradiation. The membranes were incubated for 6 h at 42°C in a prehybridization buffer consisting of 50% deionized formamide. 5 X SSPE, 5 X Denhardt's solution, 0.5% (w/v) SDS and 100 μg/ml of denatured salmon sperm DNA, and then hybridized with 2×10^6 cpm/ml of ^{32}P-labeled DNA probes for 20 hr. After hybridization, membranes were washed twice with 2 X SSPE containing 0.1% SDS for 15 min, twice with 1 X SSPE containing 0.1% SDS for 10 min and 0.1 X SSPE with 0.1% SDS for 10 min at 42°C. Membranes were then exposed to x-ray films at −80°C with intensifying screens. Autoradiographs were scanned by the computerized densitometry as described above for gel-scanning.

Zymography. Growing and regressing PDMT were analyzed by zymography according to the method of Chin *et al.* (41) with some modifications. SDS-polyacrylamide gels containing 1 mg/ml casein were prepared with 1.3 mg/ml ammonium persulfate. Samples were mixed with a buffer containing 4% SDS, 125 m*M* Tris-HCl (pH 6.8), and 10% glycerol, and then electrophoresed on the casein-containing gels. After electrophoresis, the proteinases separated on the gels were renatured by gently shaking the gels in 50 m*M* Tris-HCl (pH 7.5) containing 2.5% Triton X100 and 0.1 *M* NaCl for 1 hr at room temperature, followed by incubation in 50 m*M* Tris-HCl (pH 7.5) containing 10 m*M* $CaCl_2$ for 18 hr at 37°C. The resultant gels were stained with Coomassie brilliant blue.

Immunoblot Analysis of Matrilysin. Proteins resolved by electrophoresis were transferred to PVDF membranes and incubated in Tris-buffered saline (TBS; 10 m*M* Tris-HCl [pH 7.4], 0.15 *M* NaCl) containing 1% skim milk at 37°C overnight. The membranes were then treated with rabbit anti-human matrilysin antibody (kindly supplied by Dr. K. Miyazaki of Kihara Biological Institute, Yokohama City University) at 1:600 dilution for 2 hr. The membrane washed in TBS was incubated with peroxidase-conjugated goat anti-rabbit IgG (1:100 dilution; Cappel, West Chester, PA) for 2 hr. The enzyme-catalyzed reaction was induced with 0.05% diaminobenzidine and 0.03% H_2O_2 as described previously (42).

Statistics. Data were evaluated by analysis of variance (ANOVA) followed by Dunnett's multiple

range test. Experiments were performed at least three times in all studies, and values were expressed as mean ± SEM.

Results

Mitotic Indices and Areas Occupied by Epithelial and Stromal Cells of PDMT. The weight of PDMT increased rapidly until parturition and decreased soon after parturition (Fig. 1A). Mitotic indices in both epithelial and stromal cells of PDMT were higher than in epithelial and stromal cells of MG from the same animal (Fig. 1 B and C). The mitotic index in epithelial cells of PDMT reached a maximum plateau level on Day 18 of pregnancy and then decreased to approximately one-fourth of the maximum on Day 2 after parturition. Mitotic index in stromal cells of PDMT reached a maximum level on Day 19 of pregnancy and then decreased gradually from Day 20 of pregnancy to Day 2 after parturition. Mitotic index in stromal cells of pregnancy-independent MT could not be determined, since few stromal cells were found in MT. The areas occupied by epithelial and stromal cells of PDMT were then measured. The areas occupied by stromal cells of PDMT on Day 15, 18, and 20 of pregnancy and on Day 0, 1, and 2 after parturition were significantly lower than those occupied by the epithelial cells (Fig. 2).

PDMT in Hormone-Treated Mice. The size of PDMT in the intact control group increased rapidly until parturition and decreased soon after parturition (Fig. 3). Treatment with E_2 failed to prevent the decrease after parturition. The maximum size of PDMT was not different between E_2-treated and control groups. In contrast to E_2, progesterone delayed parturition for 2 days. In the progesterone-treated group, PDMT continued to grow until 4 days after parturition and then gradually regressed. Removal of progesterone-pellet after parturition induced a rapid regression of PDMT.

DNA Fragmentation. DNA fragmentation, as revealed by electrophoretic analysis, is shown in Figure 4. The ladder pattern of DNA degradation into nucleosomal oligomers was observed on Day 20 of pregnancy and on the day of parturition in PDMT.

Protein Expression. The majority of total tissue proteins were present on a 13% polyacrylamide gel at pI ranging from 4.0 to 8.5 and between 14 and 94 kDa. The major changes in protein profiles of PDMT at the time of appearance (Day 15 of pregnancy), growth (Day 20 of pregnancy) and regression (on the day of parturition) are listed in Table I. Proteins showing similar changes are grouped and numbered I to VI. The major changes in protein profiles of MG (Day 15 and 20 of pregnancy and the day of parturition) and MT are also listed in Table I for comparison. On Day 20 of pregnancy, eight protein spots (Group I; nos. 1, 2, 4–7, 16, and 25) increased in intensity, and five protein spots (Group II; nos. 3, 9, 15, 17, and 24) appeared anew in PDMT, compared with those found on Day 15 of pregnancy and on the day of parturition. Five protein spots (Group III; nos. 8, 11, 14, 21, and 27) increased in intensity, and six new spots (Group IV; nos. 13, 18–20, 22, and 26) appeared in regressing PDMT (on the day of parturition). The intensity of one protein spot (Group V; no. 12) increased on Day 20 of pregnancy and on the day of parturition. Three proteins

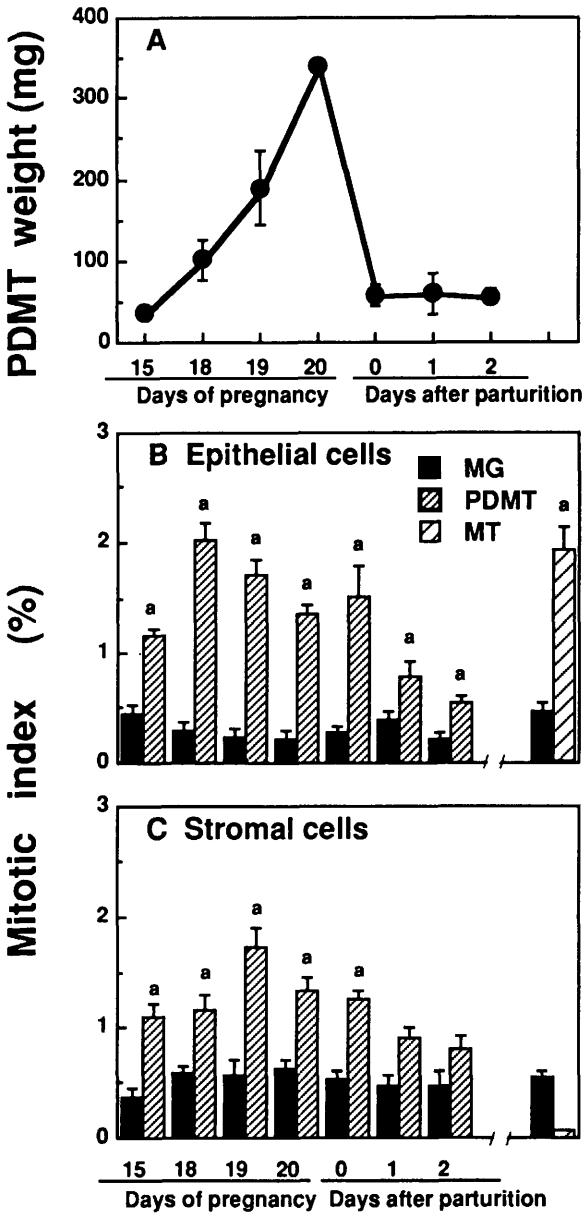

Figure 1. Tumor weights (A) and mitotic indices in epithelial (B) and stromal cells (C) of PDMT during pregnancy and of autonomous mammary tumors (MT). MG indicates normal mammary gland. Data are expressed as mean ± SEM of eight samples. [a]Significant differences compared with those of MG at $P < 0.001$ (analysis of variance followed by Dunnett's multiple range test).

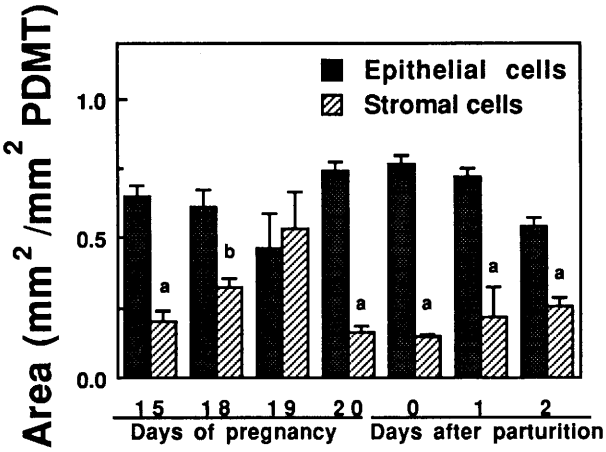

Figure 2. Area occupied by epithelial cells and stromal cells of PDMT during pregnancy. Data are expressed as mean ± SEM of eight samples. [a,b]Significant differences compared with those of epithelial cells at $P < 0.001$ and 0.05, respectively.

(Group VI; nos. 10, 23, and 28) decreased their intensity on the day of parturition. Actual polypeptide patterns of PDMT on Day 15 and 20 of pregnancy and on the day of parturition are shown in Figure 5.

Fourteen protein spots (nos. 2, 4, 8, 10, 11, 15, 17–20, 23, 24, 26, and 27) and one protein spot (no. 1) were present only in epithelial cells and stromal cells of PDMT, respectively (Table I). However, seven protein spots (nos. 5, 6, 12, 21, 22, 25, and 28) were expressed both in epithelial and stromal cells of PDMT (Table I). In epithelial cells, two proteins (nos. 2 and 4) showed intense expression, and three proteins (nos. 15, 17, and 24) appeared newly on Day 20 of pregnancy. On the day of parturition, three proteins (nos. 8, 11, and 27) were intensely expressed, four proteins (nos. 18–20 and 26) appeared newly, and two proteins (nos. 10 and 23) showed decreased expression. In stromal cells, one protein (no. 1) showed an intense expression on Day 20 of pregnancy. Both in epithelial cells and stromal cells of PDMT three proteins (nos. 5, 6, and 25) were intense in expression on Day 20 of pregnancy, one protein (no. 21) showed an intense expression, and one protein (no. 22) appeared newly on the day of parturition. One protein (no. 12) was intense in expression on Day 20 of pregnancy and on the day of parturition. One protein (no. 28) showed decreased expression on the day of parturition.

In MG, one protein (no. 1 in Group I) showed an intense expression on Day 15, and one protein (no. 17 in Group II) appeared newly on Day 20 of pregnancy. Seven protein spots (nos. 8, 11, and 27 in Group III and nos. 13, 18, 20, and 22 in Group IV) appeared newly on the day of parturition. Two protein spots (no. 7 in Group I and no. 14 in Group III) increased their intensity in MG of 6- to 7-month-old mice (Table I).

When the polypeptide patterns of PDMT and MG were compared on Day 20 of pregnancy and on the day of parturition (Fig. 6 and Table I), it was found that four polypeptide spots (nos. 9, 15, 19, and 24) were present in PDMT but not in MG.

Protein Expression of PDMT in Progesterone-Treated Mice. Expression of PDMT proteins in progesterone-treated mice is listed in Table I. No new protein was found in growing PDMT (Day 3 after parturition); however, two protein spots (nos. 17 and 24) appeared newly in regressing PDMT (Day 6–7 after parturition). Three protein spots (nos. 8, 11, and 22) appeared newly in regressing PDMT one day after removal of progesterone pellets.

Amino Acid Sequence Analysis. The protein no. 24 newly expressed on Day 20 of pregnancy in PDMT, and the proteins nos. 20 and 22 newly expressed on the day of parturition in PDMT were analyzed on a gas-phase sequencer for their N-terminal

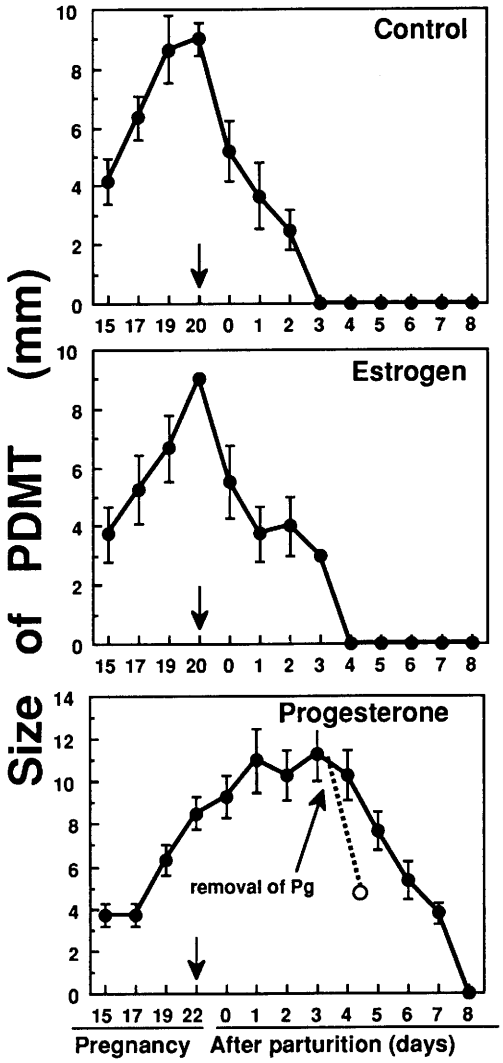

Figure 3. The size of PDMT in mice treated with E_2 pellet or progesterone pellet during pregnancy. Female mice bearing PDMT on Day 15 of the second pregnancy were given one E_2 or three progesterone pellets. Control mice were given three cholesterol pellets. Ten mice were used in each group. Arrows indicate the days of parturition.

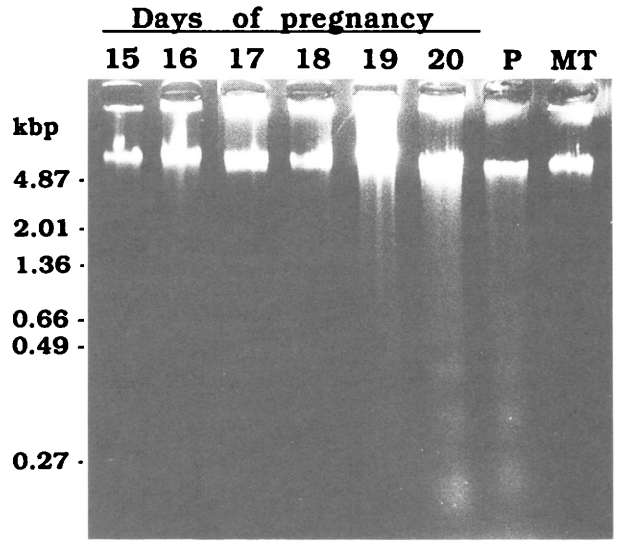

Figure 4. Electrophoretic analysis of DNA fragmentation in PDMT during pregnancy and in autonomous mammary tumors. Numbers 15–20 indicate PDMT on Day 15–20 of pregnancy; P = PDMT on the day of parturition; MT = autonomous mammary tumors.

amino acid sequences. The N-terminal amino acid sequences of protein nos. 15 and 26 could not be determined, since N-terminal amino acid residues were blocked. The sequences of nos. 24 and 22 were Thr-Glu-Leu-Thr-Lys-Cys-Lys-Val-Ser-His-Ala-Ile-Lys-Asp-Ile and Thr-Glu-Leu-Thr-Lys-Cys-Lys-Val, respectively. The N-terminal sequences of these two proteins were identical to that of α-lactalbumin (number of residues: 123; MW: 14.0 kDa; position: 1–15 and 1–8) by searching the database. The N-terminal sequence of no. 20 was Val-Phe-Leu-Thr-Val, and this matched that of eight proteins: arylsulfatase from *sea urchin* (position 485–489), chitin synthase 2 from yeast (position 969–973), cytochrome C oxidase polypeptide from *C. elegans* (position 199–203), glucose-6-phosphate 1-dehydrogenase (G6PD) from yeast (position 131–135), maltose transport inner membrane from *E. coli* (position 292–296), probable L1 protein from human papillomavirus type 2 A (position 340–344) and also from *Rhesus* papillomavirus type 1 (position 179–183) and hypothetical protein in gerd 5′ region from *Bacillus subtilis* (position 73–77).

mRNA Expression of Proto-Oncogenes and ER. To determine mRNA expression patterns of proto-oncogenes, c-*jun*, c-*fos*, and c-*myc*, and estrogen receptor, total RNA was isolated from PDMT on Day 15 and 20 of pregnancy and on the day of parturition and from MT. Northern blot analysis was performed using v-*jun*, v-*fos*, v-*myc*, estrogen receptor, and human GAPDH cDNA probes. A quantitative

Table I. Properties of Proteins in Pregnancy-Dependent Mammary Tumors (PDMT), Mammary Glands, and Autonomous Mammary Tumors (MT) in GR/A Mice and in Mice Receiving Progesterone (Pg) Pellets

Group No.	Protein No.		MW (kDa)	pI	PDMT			MT	Mammary glands				Pg treated PDMT		
					15	20	P		15	20	P	Retired	Growth[a]	Regression[b]	Pg(−)[c]
	1	S	47	6.0	++	+++	+	++	+++	++	+	+	+	+	+
	2	E	44	7.7	++	+++	+	−	+	+	++	+	++	++	+
	4	E	38.5	5.6	++	+++	−/+	++	+	++	+	+	++	++	+
	5	S, E	38.5	6.4	+	++	+	+	+	+	+	−	++	+++	+
I	6	S, E	35	5.65	+	+++	−/+	++	+	+	−	+	+	++	+
	7		30	8.3	+	+++	+	+	+	+	+	+++	+	++	+
	16		20	5.6	+	+++	+	−	+	+	++	−	++	+	+
	25	S, E	16	5.8	++	+++	+	+	+	++	+	+	+	++	++
	3		44	7.9	−	+++	−	−	−	+	+	+	+	+	−
	9		29	6.5	−	++	−	−	−	−	−	−	−	+	+
II	15	E	20	5.8	−	+++	−	−	−	−	−	−	−	++	++
	17	E	20	7.0	−	−/+	−	−	−	+	−	−	−	+	−
	24	E	16	5.5	−	++	−	−/+	−	−	−	−	−	+	−
	8	E	27	7.4	+	−/+	+++	−	−	−	+++	−	−	−	+
	11	E	23	6.6–7.0	+	+	++/+++	−	−	−	+++	−	−	−	+
III	14		21	5.7	+	+	+/+++	−	+	+	+	++	+	+	++
	21	S, E	17	5.5	−	−/+	++	+	−	+	+	−	−	+	+
	27	E	14.5	6.5	−/+	−/+	+++	−	−	−	++	−	−	−	−
	13		22.5	6.3	−	−	+	−	−	−	+	−	−	−	−
	18	E	19.5	6.7	−	−	−/+	−	−	−	−/+	−	−	−	−
	19	E	18.5	6.7	−	−	+/+++	−	−	−	−	−	−	−	−
IV	20	E	17.5	6.7	−	−	−/++	−	−	−	−/++	−	−	−	−
	22	S, E	16.5	5.7	−	−	++	−/+	−	−	+++	−	−	−	++
	26	E	14.5	7.0	−	−	+++	−	−	−/+	+++	−	++	+	+
V	12	S, E	23	5.9	+	+++	+++	+	+	++	++	+	+	+	+
	10	E	28.5	6.0	++	+++	−/+	−	−	−/+	−	−	−	+	++
VI	23	E	16.3	6.3	++	+	+	+	++	+	+	+	++	+	+
	28	S, E	14.5	6.0	++	++	+	+	+	++	++	++	+	+	+

Note. −, +, ++, and +++ indicate relative O.D. <10, 11–30, 31–60, and 61<, respectively (actin O.D. = 100). P = the day of parturition. The proteins marked S and E were expressed in stromal and epithélial cells of PDMT, respectively. Proteins showing similar changes are grouped and numbered I to VI.

[a] Day 3 after parturition.

[b] Day 6–7 after parturition.

[c] One day after Pg removed.

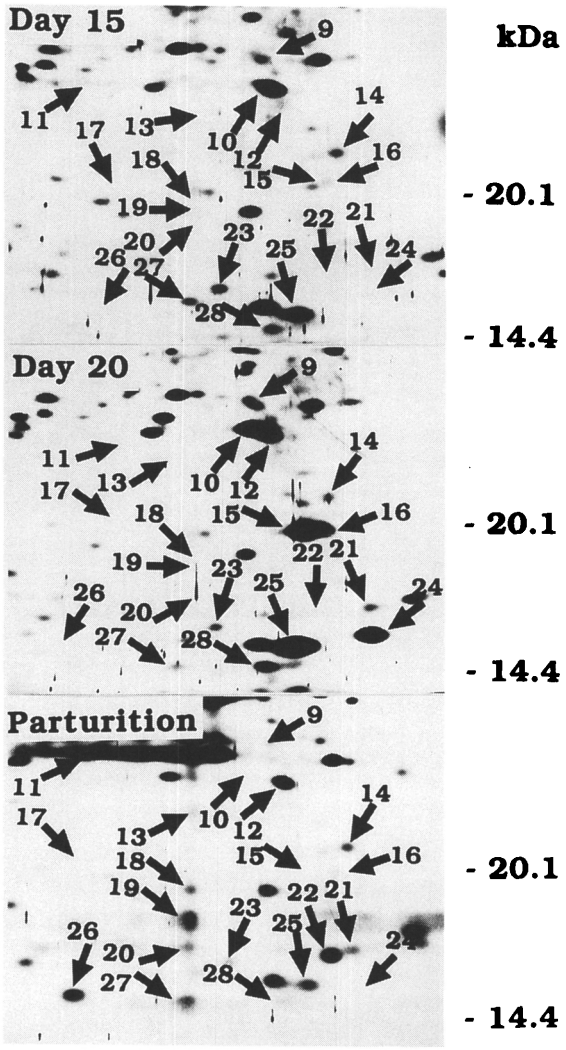

Figure 5. Two-dimensional electrophoretic patterns of 14.4- to 29.0-kDa proteins in PDMT on Day 15 and 20 of pregnancy and on the day of parturition. Numbers on figures represent individual proteins changed from Day 15 and 20 of pregnancy, and just after parturition. Experiments were repeated three times and similar results were obtained.

densitometric scanning of the autoradiographic signals was normalized on the basis of GAPDH mRNA concentration in the same blot, and is shown in Figure 7. Densitometric analysis of the hybridization revealed that the expression of c-*jun* mRNA in PDMT decreased on Day 20 of pregnancy. The expression of c-*jun* mRNA in PDMT on the day of parturition and in MT indicated 50% and 130% increase, respectively, when compared with that of Day 15 of pregnancy in PDMT. The expression of c-*fos* mRNA in PDMT showed 50% and 30% decrease on Day 20 of pregnancy and on the day of parturition, respectively, when compared with that on Day 15 of pregnancy. The expression of c-*fos* mRNA in MT was the same as that on Day 15 of pregnancy. There was a slight increase in the expression of c-*myc* mRNA in MT but no difference was observed in various stages of PDMT. The estrogen receptor mRNA expression in PDMT showed a marked reduction on Day 20 of pregnancy (40%) and on the day of parturition (50%) when compared with that on Day 15 of pregnancy. Estrogen receptor mRNA expression in MT was the lowest, being 25% of that of PDMT on Day 15 of pregnancy.

Zymography. PDMT on Day 15 and 20 of pregnancy and on the day of parturition secreted 29- and 19-kDa metalloproteinases. From Western blotting, these proteinases were identified as matrilysin (29 kDa; proenzyme, 19 kDa; active-form) (Fig. 8). They

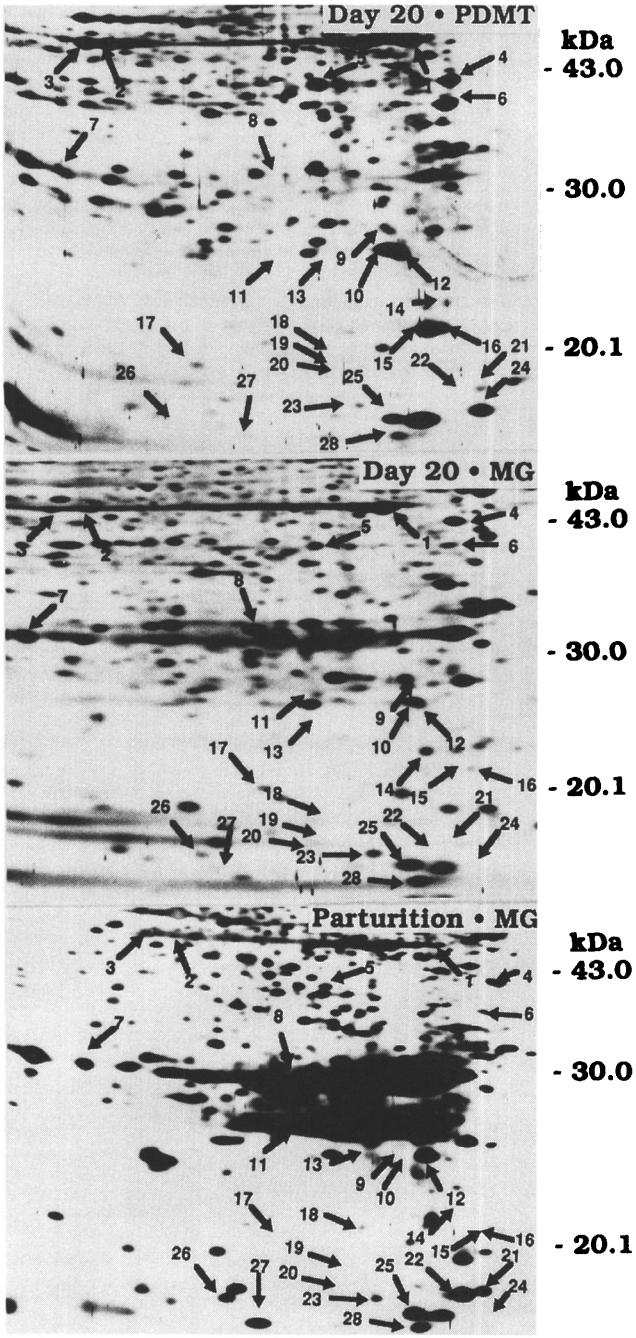

Figure 6. Two-dimensional electrophoretic patterns of 14.4- to 43.0-kDa proteins in PDMT and MG on Day 20 of pregnancy.

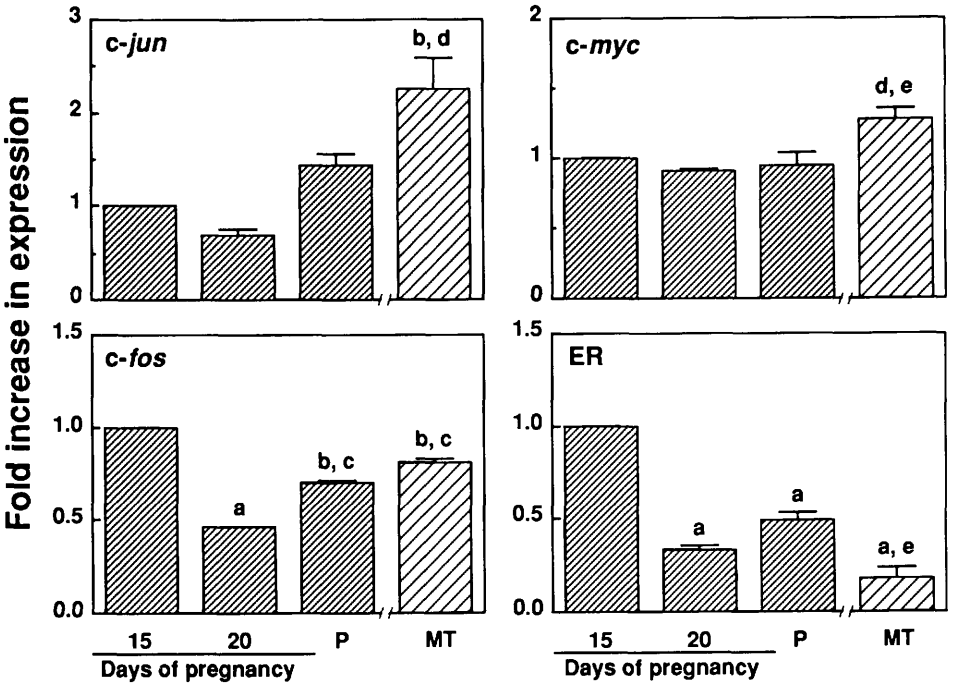

Figure 7. Densitometric analyses of expression of c-*jun*, c-*fos*, c-*myc*, and estrogen receptor (ER) mRNAs in PDMT during pregnancy and in MT from the Northern blot analysis. A quantitative scanning of autoradiographic signals was normalized on the basis of GAPDH mRNA concentration in the same blot. P and MT indicate PDMT on the day of parturition and autonomous mammary tumors, respectively. [a,b]Significantly different from Day 15 of pregnancy: [c,d]significantly different from P. $^{a,c}P < 0.01$; $^{b,d,e}P < 0.05$.

increased in intensity in PDMT on Day 15 of pregnancy and on the day of parturition as compared with PDMT on Day 20 of pregnancy. MT did not secrete these proteinases.

Discussion

PDMT appeared on Day 15 of pregnancy in GR/A mice and grew rapidly until Day 20 of pregnancy and regressed on the day of parturition as reported previously (1–3). That PDMT are hormone-dependent mammary tumors (4–7, 9) is also shown in this study. The growth of PDMT in progesterone-treated mice was greater than that in E_2-treated mice, indicating that the growth of PDMT depends on the presence of progesterone. The mitotic indices of epithelial cells and stromal cells of PDMT began to decrease gradually from Day 18 and 19 of pregnancy, respectively. The area occupied by stromal cells increased until Day 19 of pregnancy and then rapidly decreased on Day 20 of pregnancy. In contrast, the area occupied by epithelial cells reached a plateau on the day of parturition and then decreased gradually. These results indicate that the rapid decrease in stromal cells on Day 20 of pregnancy is reflected in the rapid regression of PDMT. Since DNA fragmentation, an apoptosis indicator, was observed in PDMT on Day 20 of pregnancy and on the day of parturition, it was conjectured that the initial rapid regression of PDMT was caused by the apoptosis of stromal cells and subsequent reduction of area occupied by stromal cells. Apoptotic cells in PDMT determined by the terminal transferase end-labeling technique increased *ca.* 10-fold on Day 20 and on the day of parturition compared with Day 17 of pregnancy (unpublished data), supporting the DNA fragmentation.

In previous studies, some proteins were shown to be newly induced in growth and regression of rat prostate (13–16), human mammary cancer (17–19), and rat uterine cells (23). Estrogen regulates secretion of procathepsin D, cathepsin D, and TGF-α and -β (17–20). Procathepsin D and TGF-α stimulate growth of breast cancer cells and uterine cells *in vitro* (18, 20, 21) but cathepsin D and TGF-β inhibit their growth (18, 19). Growth inhibitors of mammary gland cells and breast

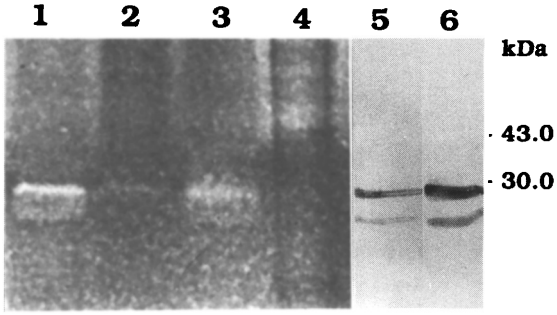

Figure 8. Zymographic patterns in casein gel and Western blot analysis of extracts of PDMT and MT. Lane 1–3, zymographs of PDMT extracts on Day 15 and 20 of pregnancy and on the day of parturition; Lane 4, zymograph of MT extract; lane 5, Western blot analysis of PDMT extract on Day 15 of pregnancy using anti-matrilysin antibody; Lane 6, Western blot analysis of purified matrilysin.

carcinoma cells *in vitro* have been purified (22–25). In the regressing prostate after androgen withdrawal, cathepsin D was also induced (16). In the present study, we detected new expression of 3 PDMT-specific proteins (29, 20 [pI 5.8], and 16 kDa) on Day 20 of pregnancy (the day on which apoptosis occurs). The 16 kDa protein was identified as α-lactalbumin by N-terminal amino acid sequencing. Since 29-kDa protein was not expressed in epithelial cells and stromal cells of PDMT, this protein may be from extracellular matrix. Therefore, one protein (20 [pI 5.8] kDa, no. 15) may be associated with the regression of PDMT. This protein could not be determined, since N-terminal acid residues were blocked. Four proteins (27, 23, 17, and 16.5 kDa) in regressing PDMT also appeared on the day of parturition in both PDMT and MG. Sixteen and five-tenths kiloDalton protein (no. 22) was identified as α-lactalbumin by amino acid sequencing, suggesting that these proteins (27, 23, and 16.5 kDa) were expressed under the conditions where progesterone levels were low.

Expressions of c-*jun*, c-*fos*, and c-*myc* mRNAs are stimulated by estrogen in rat uterus (43–46). The activation of c-*fos* and c-*jun* genes in rat uterus is due to an increase in transcription and is not prevented by protein synthesis inhibitors; thus, the activation of c-*fos* and c-*jun* genes is a primary response to the estrogen receptor complex (47, 48). PDMT and MT are hormone-dependent and -independent mammary tumors, respectively (8). In the present study, estrogen receptor mRNA expression in PDMT was high on Day 15 of pregnancy (the appearance of PDMT) but low in MT. The expression pattern of c-*fos* mRNA in PDMT was similar to that of estrogen receptor mRNA. These results suggest that the growth of PDMT is under hormonal control and the increase in c-*jun* and c-*fos* genes is involved in the appearance and growth of PDMT. The growth of MT is autonomous of hormonal control. *myc* induces apoptosis in several cell lines *in vitro* (49, 50); however, no difference in c-*myc* expression was observed at various stages of PDMT in the present study.

Activities of matrix metalloproteinases are elevated in developing prostate (26) and some tumor cell lines (27–31). Stromelysin-3 mRNA is expressed during apoptosis of MG cells after weaning and of fibroblastic cells of breast carcinoma, so that stromelysin-3 is implicated in extracellular matrix remodeling following mammary apoptosis and breast cancer progression (32). Matrilysin, one of matrix metalloproteinases, hydrolyzes fibronectin, laminin, and collagens including type IV collagen (28). Many matrix metalloproteinases are secreted by various cell types. However, matrilysin is secreted only by restricted types of cells such as human rectal adenocarcinoma cell line CaR-1, human breast cancer, and colon cancer (28, 29). The presence of matrilysin can be demonstrated immunohistochemically in the basal lamina when matrilysin gene-transfected prostate cancer cells are transplanted into mice. Tumor invasion is significantly higher in the matrilysin gene-transfected cells than that in mock-transfected cells (51). Matrix metalloproteinases are thought to be required for tumor invasion (27, 29, 30, 51). PDMT are benign tumors, whereas MT are malignant tumors (8). In the present study, matrilysin was produced in greater amount at initiation and regression stages of PDMT than at the growing stage; however, no detectable amounts of matrilysin were present in MT.

In conclusion, these findings show that the growth of PDMT is under hormonal control, resulting in increased expression of c-*jun*, c-*fos* and ER genes, that apoptosis occurs in the regression of PDMT on Day 20 of pregnancy, and that this regression is associated with a newly expressed protein (20 kDa, pI 5.8) on Day 20 of pregnancy. Matrilysin expression may be associated with both the initiation and regression of PDMT.

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