Expression of collagenase-3 in the rat ovary during the ovulatory process

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Abstract

We have examined the expression of the murine counterpart of human collagenase-3, a matrix metalloproteinase produced by breast carcinomas, in the course of processes which involve extensive tissue remodeling. By using Northern blot analysis, we have found that collagenase-3 is expressed in the rat ovary, but not in the remaining analyzed tissues including brain, kidney, liver, lung, mammary gland, uterus, bladder, heart, intestine, prostate, spleen, testis and thymus. Collagenase-3 mRNA was detected at high levels in rat ovaries at proestrus and estrus, was at a minimum at metestrus and started to increase during diestrus through to proestrus. In addition, collagenase-3 was also detected on day 21 of pregnancy, which is approximately one day before parturition. However, no significative expression was detected in RNA from ovaries taken immediately after parturition, or on days 1, 5 or 30 postpartum. Northern blot analysis also revealed that collagenase-3 was not expressed at significant levels, compared with ovarian expression, in the uterus or in the mammary gland during pregnancy or after parturition. When follicular granulosa cells were separated from Northern blot, it was seen that collagenase-3 was not expressed by the granulosa cells but was present in the residual tissue containing interstitial and thecal tissues, growing follicles and corpora lutea. Immunohistochemical studies also confirmed, at the protein level, the localization of collagenase-3 in rat ovary. Gonadotropic stimulation of ovulation in immature rats by priming with pregnant mare's serum gonadotropin and stimulation with human chorionic gonadotropin failed to induce the expression of collagenase-3, suggesting that additional factors which are not present in the immature stimulated rats are needed for completely effective induction of the expression of this matrix metalloproteinase. On the basis of these results, together with the comparative analysis of expression of different matrix metalloproteinases in the rat ovary, we propose that collagenase-3 is a major ovarian metalloproteinase potentially involved in ovarian function during the reproductive cycle.

Introduction

Matrix metalloproteinases (MMPs) form a family of structurally related enzymes with ability to degrade different components of the extracellular matrix (Woessner 1991, Matrisian 1992, Murphy & Docherty 1992, Birkedal-Hansen et al. 1993). According to structural and functional properties, the family of mammalian MMPs consists of at least three different groups of closely related members: collagenases, stromelysins and

gelatinases. All of them are similar in that they are secreted as latent proenzymes, contain several conserved domains with apparent specific functions including an activation locus and a zinc-binding site, and can be inhibited by tissue-specific inhibitors and chelating agents. However, they differ in their specificities towards extracellular matrix fibronectin (Nagase et al. 1991). Recently, we have identified and characterized a novel human member of this family of proteolytic enzymes which has been designated collagenase-3 since it represents the third member of this subfamily of MMPs (Freije et al. 1994, Pendas et al. 1995). The human collagenase-3 displays the same domain organization as the remaining members of the family and has the substrate specificity on fibrillar collagens anticipated from its amino acid sequence (Freije et al. 1994). The collagenase-3 gene is expressed by human mammary carcinomas but not by the normal resting mammary gland nor by a number of different human tissues, which has led us to suggest that this proteinase may play a role in the lytic processes associated with breast cancer progression (Freije et al. 1994). However, the absence of detectable collagenase-3 expression in normal human tissues has hampered studies to elucidate the physiological function of this enzyme. One likely possibility to explain the absence of collagenase-3 expression in normal tissues is that this proteolytic enzyme is only produced at specific moments by tissues undergoing remodeling or breakdown events, such as those occurring in several reproductive processes. In fact, previous studies from different groups have detected the expression of diverse MMPs in processes such as embryo implantation (Librach et al 1994, Polette et al. 1994), uterine cervix dilatation during parturition (Rajabi et al. 1988, Rechberger & Woessner 1993), uterine post¬partum involution (Roswit et al. 1988, Wilcox et al. 1992), ovulation (Reich et al. 1985, Palotie et al. 1987, Curry et al. 1988, 1989) and mammary gland involution (Lefebvre et al. 1992, Talhouk et al. 1992). If we consider that collagenase-3 expression by breast carcinomas could be the result of hormonal alterations, then this enzyme could also be produced in the course of those processes specific to reproduction and highly regulated by hormones.

On this basis, and taking advantage of the fact that a rat homolog of collagenase-3 (also called rat interstitial collagenase) has been characterized previously (Quinn et al. 1990), we have addressed the question of the putative role of collagenase-3 in reproductive processes by using this animal model. In this study, we show that collagenase-3 is produced by the rat ovary with striking variations during the estrous cycle. We also use immunohistochemical techniques to localize the cellular origin of collagenase-3. Finally, by performing a comparative analysis of expression of different MMPs in the rat ovary, we propose that collagenase-3 is a major ovarian metalloproteinase potentially involved in the ovulation process.

Materials and Methods

Experimental animals and treatments

Female Wistar rats were used. They had free access to water and standard rat pellets were available ad libitum (Panlab S.L., Barcelona, Spain). The animals were kept under regulated light/darkness conditions (12 h light, 12 h darkness, cycles starting at 0800 h), constant temperature (22-24 °C) and relative humidity (60-70%). Mature 3-month-old rats were assessed for stage of the estrous cycle by daily examination of vaginal smears. After at least three consecutive regular cycles, the animals were killed by decapitation on diestrus day 1, diestrus day 2, proestrus, estrus and metestrus. Ovaries and other tissues of interest including brain, kidney, liver, lung, mammary gland, uterus, bladder, heart, intestine, prostate, spleen, testis and thymus, were removed, quickly frozen and stored in liquid nitrogen before processing for RNA purification. Estrous cycles were also monitored in old female rats (16—18 months old). These analyses indicated that the rats were in a state of constant estrus, pseudopregnancy or irregular estrous cyclicity. Ovaries were collected from these old acyclic rats and from pregnant rats at various stages of gestation (day 1 being the day of appearance of a copulation plug) as well as from rats on days 1, 5 and 30 postpartum. In some experiments rats were weaned at 9 days postpartum (Talhouk et al. 1992) and ovarian samples were collected at 3, 6 and 20 days post-weaning. In all cases, ovaries were collected in the morning of the different estrous cycle stages. To stimulate follicular development, immature rats (26 days old) were injected s.c. in the morning with a single dose of 15 IU pregnant mare's serum gonadotropin (PMSG) (Sigma Chemical Co., St Louis, MO, USA) diluted in saline. Forty-eight hours after PMSG treatment, the animals were injected i.p. with 6 IU human chorionic gonadotropin (hCG; Sigma Chemical Company) to induce ovulation. The animals were killed by cervical dislocation 0, 1,3, 6, 9, 12, 24, 48 and 72 h after hCG injection. The ovaries were removed and processed as described elsewhere. All samples were taken at 1000 h and experiments were replicated at least three times.

Granulosa cell isolation

Granulosa cells were isolated essentially as described by Mann et al. (1991). Briefly, ovaries were placed in PBS, the largest Graafian follicles were punctured with a fine needle, and the granulosa cells were collected, washed by centrifugation and processed for RNA extraction. The residual tissue containing interstitial and thecal tissues, growing follicles as well as corpora lutea was also processed for RNA purification.

PCR amplification of rat collagenase-3 RNA

cDNA synthesis was carried out with the RNA-PCR Kit from Perkin-Elmer Cetus (Foster City, CA, USA). The reverse transcription was performed for 30 min at 42 °C with 1 µg total ovary RNA. The whole mixture was used for PCR amplification with the following oligonucleotides deduced from cloned rat collagenase (Quinn et al. 1990): 5'ATTGTGAACTACACCCCTG and 5'TGGCCAAG CTCATGGGCA. Conditions for the amplification were 40 cycles of 45 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C. The 314 bp amplified fragment was cloned into pBluescript (Stratagene, Heidelberg, Germany),

checked by DNA sequence and used as the probe for collagenase-3. The nucleotide sequence of the PCR-generated probe from ovarian tissue was identical to the sequence reported for rat interstitial collagenase from osteosarcoma cells (Quinn et al. 1990).

Northern blot analysis

Total RNA from the different rat tissues was isolated by guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi 1987). Samples of about 20 µg total RNA were separated by electrophoresis in 1-2% agarose-formaldehyde gels and blotted onto Hybond nylon filters (Amersham International, Amersham, Bucks, UK). Filters were prehybridized at 42 °C for 3 h in 50% formamide, 5 x SSPE (1x =150 mM NaCl, 10 mM NaH_2P0_4, 1 mM EDTA, pH 7-4), 2 x Denhardt's, 0-1% SDS and 100 µg/ml denatured herring sperm DNA and then hybridized for 24—48 h under the same conditions. Probes were labeled with [α^{32}P]dCTP (3000 Ci/mmol) by random priming with the Rediprime kit (Amersham International). Filters were washed with 2 x SSC, 0-05% SDS for 3 x 15 min at room temperature and then with 0-1 x SSC, 0-1% SDS for 2 x 20 mm at 50 °C and exposed to autoradiography at — 70 °C. Filters hybridized with collagenase-3 probes were exposed during 5-7 days while those hybridized with other MMP probes were exposed for at least two weeks. When the probes were of human origin instead of rat, the second washing step with 0-1 x SSC and 0-1% SDS was substituted by 2 x SSC, 0 5% SDS at 50 °C.

Immunohistochemical analysis

Rat ovaries were fixed by immersion with 4% para¬ formaldehyde in PBS for 5 h at 4 °C, embedded in paraffin and cut into 5 µm thick sections. After routine deparaffinization, sections were treated with 0-3% H_20_2 in methanol for 30 min to block endogenous peroxidase activity, washed with PBS and incubated with 1% normal serum for 30 min. Immunohistochemical assays were performed using the avidin-biotin procedure. Sections were incubated in a moist chamber for 12 h at 4 °C with antiserum against rat collagenase-3 diluted 1:500 (kindly provided by Dr G Lyons, University of Sydney, Australia). The characteristics of this polyclonal antibody have been described previously (Whitelock et al. 1989). Then, the slides were incubated sequentially with biotinylated goat anti-rabbit antibody (Dako, Copenhagen, Denmark) and peroxidase-conjugated streptavidin (Biomeda Corp., Foster City, CA, USA) for 30 min each at room temperature in a moist chamber. After washing with PBS, the reaction was developed by incubation with a solution containing 0-66 mM 3,3'-diaminobenzidine and 2 mM H_20_2 in 50 mM Tris—HC1, pH 7.6. Sections were finally counterstained for nuclei with hematoxylin, dehydrated and mounted with Eukitt. Specificity of staining was determined by incubating tissue sections alone or with an equal amount of IgG from non-immunized rabbits.

Results

In order to examine the expression of the collagenase-3 gene in rat tissues, Northern blots containing total RNAs obtained from many different tissues were hybridized with a PCR-generated probe corresponding to a 314 bp segment of rat collagenase-3 cDNA. As shown in Fig. 1, a clear hybridizing band of about 2-9 kb was detected in RNA isolated from rat ovary. By contrast, we did not detect any positive signal of collagenase-3 expression in the remaining analyzed tissues including brain, kidney, liver, lung, mammary gland, uterus, bladder, heart, intestine, prostate, spleen, testis and thymus. It is also noteworthy that collagenase-3 expression was undetectable in brain, kidney, liver and lung from either male or female animals (Fig. 1 and data not shown).

The occurrence of collagenase-3 expression in rat ovary strongly suggested that this matrix metalloproteinase could be involved in any of the extracellular matrix remodeling processes occurring within the ovary during the ovulatory process. To address this question further, we investigated whether collagenase-3 expression was associated with any particular stage of the estrous cycle. Total RNAs were extracted from whole ovaries at different phases of the cycle and analyzed by Northern blot as above. As can be seen in Fig. 2, collagenase-3 mRNA was detected at high levels at proestrus and estrus, was at a minimum at metestrus, and started to increase during diestrus through to proestrus. It should be noted that ovarian collagenase-3 levels of expression correlate well with plasma values of estradiol during the estrous cycle, which also reach a maximum at proestrus and a minimum at metestrus (Bergman et al. 1992). Since these data pointed to a relationship between collagenase-3 expression and ovarian function, we also examined the expression of this proteolytic enzyme in rat ovaries from either immature rats or old acyclic animals (older than 16 months). As shown in Fig. 2, collagenase-3 mRNA was not detected in any of these conditions. We next examined the expression of this proteolytic enzyme during pregnancy and after parturition. For this purpose, we initially prepared total RNAs from the whole ovaries of pregnant rats on days 5, 15 and 21 and the expression of collagenase-3 was examined by Northern blot using the same probe as above. As can be seen in Fig. 3, collagenase-3 expression was only detected on day 21, which is approximately one day before parturition. However, no significative expression was detected in RNA from ovaries taken immediately after parturition, or on days 1, 5 or 30 postpartum, but it could be observed again 6 days after weaning (Fig. 3), coincident with the recovery of ovarian cyclic function. These results contrast with the expression of other metalloproteinases which could also be involved in ovarian function and whose simultaneous production appears to be essential for the efficient turnover of all connective tissue barriers present in ovarian tissues. Thus, hybridization of the same filter with a probe corresponding to mouse 72 kDa type IV collagenase (Reponen et al. 1992) showed a weak but constant expression of this metalloproteinase through all the pregnancy stages and after parturition, displaying a slight increase 1 day postpartum. Similar results were obtained when the filters were hybridized with a probe corresponding to the human interstitial collagenase 1 (MMP-1) cDNA (Goldberg et al. 1986). Thus, although at present no definitive evidence of occurrence of a rat homolog of human MMP-1 is available, a clear hybridizing band of about 1 -8 kb and distinct from

the 3 kb band corresponding to collagenase-3 (MMP-13), was detected in rat ovaries during pregnancy and after parturition. Finally, the same filters were hybridized with probes corresponding to other representative members of the matrix metalloproteinase gene family. As shown in Fig. 3, hybridization with a specific probe for stromelysin-2 (Muller et al. 1988) only gave a positive signal in the RNA corresponding to the normal proestrous ovary. Hybridization of similar filters with a stromelysin-1 cDNA probe (Whitham et al. 1986) did not give any detectable signal (not shown). It is also remarkable that filters hybridized with collagenase-3 probes were exposed to autoradiography for 5—7 days, while those hybridized with probes for other MMPs were exposed for at least two weeks. Taken together, these results indicate that collagenase-3 is a matrix metalloproteinase expressed in rat ovary at higher levels and in a more specific and controlled fashion than other members of this protein family, and provides additional evidence for our proposal that this enzyme could play a major role in ovarian function during the reproductive cycle.

In order to evaluate if collagenase-3 is also present in other tissues which undergo extensive remodeling during reproductive processes, we analyzed its expression in rat uterus and mammary gland during the normal estrous cycle, pregnancy and postpartum. Northern blot analysis revealed that collagenase-3 was not expressed at significant levels, compared with ovarian expression, in the uterus or in the mammary gland at different stages of the estrous cycle. Collagenase-3 expression was also undetectable in these tissues on different days during pregnancy and after parturition (Fig. 4 and data not shown). By contrast, when the same filters were hybridized with a probe specific for matrilysin, a clear hybridizing band of about 1 kb was detected in samples from uterus at 1 day postpartum, thus confirming previous studies on the relevance of this MMP in the process of uterine postpartum involution (Wilson et al. 1995). Furthermore, it is also noteworthy that the absence of significant collagenase-3 expression in mammary gland agrees very well with results from recent studies on MMP expression during ductal and alveolar branching morphogenesis of the murine mammary gland (Witty et al. 1995). These studies have shown that some MMPs such as stromelysin-3 or gelatinase A, but not collagenase-3, are expressed in the mammary gland during these tissue remodeling processes. On the basis of these results, it seems that collagenase-3 is mainly expressed in the rat ovary during the reproductive cycle, whereas other members of the MMP family could be responsible for the extracellular matrix remodeling events occurring in other tissues such as the uterus and mammary gland during different reproductive processes. Having shown the temporally specific pattern of collagenase-3 expression during the ovulatory process, and in an attempt to get some preliminary insights into the possible functional significance of these findings, we tried to localize more precisely the site of expression of this proteinase in the rat ovary. Follicular granulosa cells were separated from the residual ovarian tissue of ovaries taken from proestrous or estrous rats and total RNAs were extracted from both sources. Northern blot analysis (Fig. 5) revealed that collagenase-3 was not expressed by the granulosa cells but was present in the remaining tissue, this latter containing interstitial and thecal tissues, growing follicles as well as corpora lutea. The same blot hybridized with the human interstitial collagenase

cDNA probe revealed the presence ofthis mRNA in both granulosa and residual tissue, as was demonstrated previously by Reich et al. (1991), and provided additional evidence to show that the absence of collagenase-3 detectable signal in granulosa cells was not a consequence of loss of integrity of the RNA extracted from these cells. Immunohisto¬chemical studies performed with polyclonal rabbit IgGs anti collagenase-3 also confirmed, at the protein level, the above studies on localization of collagenase-3 in rat ovary. Thus, positive immunoreactivity was observed in antral follicles, interstitial gland cells as well as in the corpora lutea in the ovaries of proestrous or estrous rats, while granulosa cells in primordial follicles were devoid of immunostaining. In antral follicles, immunoreactivity was observed in theca interna cells but not in granulosa cells, whereas in corpora lutea, immunoreactivity was present in luteinized granulosa cells but not in luteinized theca cells (Fig.6 A,B,C). These cells were clearly distinguished by morphological criteria. Thus, as can be seen in the Figure, luteinized granulosa cells are large polygonal cells with abundant cytoplasm, while luteinized theca cells are small (about half the size of luteinized granulosa cells) with less abundant, darkly staining cytoplasm (Clement 1987). Furthermore, and in agreement with the above presented Northern blot analysis, no immunoreactivity was observed at all in ovaries from early pregnant or immature rats (Fig. 6 D,E). Similar results were obtained with a monoclonal antibody against rat-collagenase-3 (kindly provided by Dr G Lyons), as well as with a polyclonal antibody against human collagenase-3.

Finally, since these studies on estrous cycle stage-dependent expression of collagenase-3 in the rat ovary seemed to indicate that the gene encoding this protease was under hormonal regulation, we performed preliminary studies to evaluate the possibility that collagenase-3 mRNA induction could be correlated with gonadotropic stimulation of ovulation. We tried to reproduce experimentally the induction of follicular maturation, ovulation and corpus luteum formation in immature rats by stimulation with hCG (Richards & Bogovich 1982). Thus, immature rats were primed with PMSG for 48 h and then stimulated with hCG for 0 to 72 h. RNA was extracted from ovaries at different times and analyzed by Northern blot using a probe specific for collagenase-3. As shown in Fig. 7, collagenase-3 mRNA was not detected up to 72 h after stimulation with hCG. By contrast, control hybridization with a TIMP-1 probe revealed that, as previously reported, the levels of this tissue inhibitor of metalloproteinases in rat ovary showed a significant increase after hCG induction (Mann et al. 1991, Reich et al. 1991, Zhu & Woessner 1991). Immunohistochemical studies also failed to detect collagenase-3 in rat ovaries stimulated with PMSG and hCG (Fig. 6F and data not shown). Thus, although the experimental induction of ovulation and corpus luteum formation was effective as confirmed by TIMP-1 hybridization and by histological analysis (Fig. 6F), hCG stimulation was not sufficient for induction of collagenase-3 mRNA synthesis. Taken together, these results suggest that additional factors, which are not present in the immature stimulated rats, are needed for the completely effective induction of the expression of this matrix metalloproteinase.

Discussion

This work was originally aimed at studying the expression of collagenase-3, a matrix metalloproteinase produced by breast carcinomas (Freije et al 1994), in the course of different physiological processes which involve extensive tissue remodeling. According to the results presented herein, the murine counterpart of collagenase-3 is expressed at high levels in the rat ovary during the estrous cycle. By contrast, we did not detect significant expression of this proteolytic enzyme in other processes specific to reproduction, such as uterine postpartum involution or mammary gland involution. On the basis of these data, it is tempting to speculate that collagenase-3 might play some role in the extracellular remodeling events occurring within ovarian tissue during the reproductive cycle. The dynamics of collagenase-3 expression in rat ovary, with increasing levels found as the time of ovulation approaches, is also consistent with a potential role for this enzyme in the ovulation process.

One likely possibility to explain why collagenase-3 is expressed in the rat ovary, is that this proteinase is involved in the rupture of the follicular wall which precedes the extrusion of a mature oocyte capable of being fertilized. In fact, over the last years, studies from different groups have presented evidence on the involvement of a cascade of proteolytic enzymes, including plasminogen activators, plasmin and MMPs, in the follicular wall rupture (Reich et al. 1985, Palone et al. 1987, Curry et al. 1988, 1989, LeMaire 1989). Our finding that collagenase-3, a novel member of the MMP family displaying a strong proteolytic activity on fibrillar collagens, is highly expressed in the ovary, prompted us to re-evaluate and extend these previous studies on the relationship between MMPs and the ovulation process. The results presented herein regarding collagenase-3 expression in the rat ovary show clear differences with those concerning other MMPs, and provide further evidence that collagenase-3 (MMP-13) and interstitial collagenase (MMP-1) are distinct enzymes not only at the structural level but also in functional terms. According to Northern blot analysis and immunohistochemical studies, collagenase-3 expression was only detected to a significant extent in corpora lutea and in thecal and adjacent stromal cells but not in granulosa cells. By contrast, the mRNA hybridizing with the human interstitial collagenase probe and assumed to be MMP-1 (Reich et al. 1991) was expressed in both granulosa cells of preovulatory follicles and in the residual ovarian tissue. In addition, the comparative analysis of the relative expression of different members of this family of proteolytic enzymes has revealed that in the ovary collagenase-3 levels are much higher than those corresponding to other MMPs. Taken together, these data agree well with our proposal that this proteolytic enzyme plays a major role in the tissue remodeling events occurring during the estrous cycle. However, our failure to detect collagenase-3 could indicate that it may not have a primary role in the proteolytic degradation of the follicular wall preceding ovulation. Instead, collagenase-3 may be involved in other extracellular matrix remodeling processes occurring within the ovary in the reproductive cycle, including atresia, cumulus cell expansion and dispersion, endothelial cell migration or development of blood vessels during the massive angiogenesis that takes place in early corpus luteum formation. In fact, the finding of immunoreactive collagenase-3 in corpora lutea provides

additional support to the hypothesis that this proteolytic enzyme may be involved in some way in the formation or destruction of this extensively vascularized ovarian structure.

A final question regarding the occurrence of collagenase-3 in the rat ovary makes reference to its possible hormonal regulation. The finding described here of a localized and transient induction of collagenase-3 in ovarian tissues strongly suggests that the expression of this enzyme is a strictly regulated process. Since ovulation is under hormonal regulation, it is reasonable to think that collagenase-3 induction in the rat ovary is also mediated by hormonal factors. At present, the exact nature of the putative hormonal stimuli mediating collagenase-3 expression remains unknown although as mentioned above, it does not appear to be directly triggered by the preovulatory surge of LH. However, it should be remembered that ovulation is a complex process involving the action of factors other than gonadotropins, such as steroids, prostaglandins, growth factors and cytokines (LeMaire 1989). Interestingly, previous studies have demonstrated that some of these growth factors and cytokines, including transforming growth factor- and interleukin-1 ß, are specifically expressed in the same thecal-interstitial cells that have been shown in this work to be a source of ovarian collagenase-3 (Kudlow et al. 1987, Hurwitz et al. 1991). Further studies will be required to elucidate if some of these protein factors are responsible for inducing collagenase-3 expression by ovarian structures at specific times during the estrous cycle. It will also be of interest to examine the possibility that any of the factors with the ability to stimulate the expression of this enzyme in rat bone cells and osteosarcoma cells (Partridge et al. 1987, Clohisy et al. 1994, Varghese et al. 1994, 1995) are also effective in ovarian tissues. Finally, if we assume that overproduction of MMPs during tumor processes results from the loss of the precise controls operating in normal tissues, studies on mechanisms controlling collagenase-3 expression during the ovulatory process will also be useful in shedding some light on the mechanisms underlying its overproduction in human mammary carcinomas.

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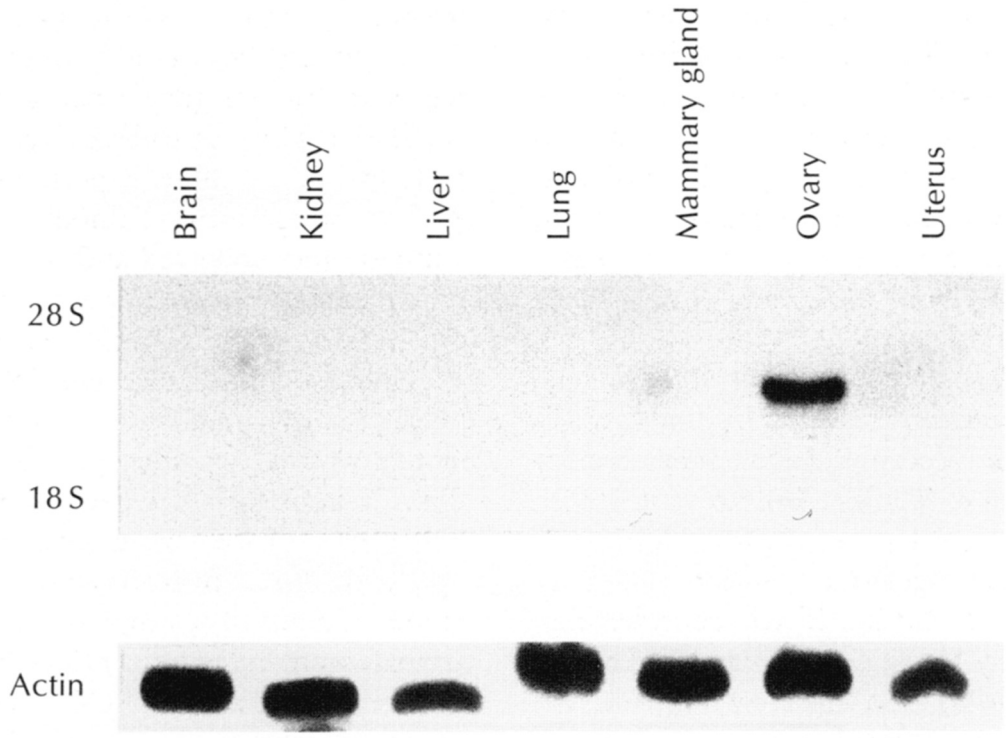

Figure 1 Expression of collagenase-3 mRNA in rat tissues. Total RNA (20 µg) was analyzed by Northern blotting using a collagenase-3 probe. Hybridization with an actin probe was performed to verify equal sample loading. Samples corresponding to ovary, mammary gland and uterus were collected from a proestrous rat. Filters were exposed to autoradiography at - 70 °C for 5 days after hybridization with the collagenase-3 probe and for 2 days after hybridization with the actin probe.

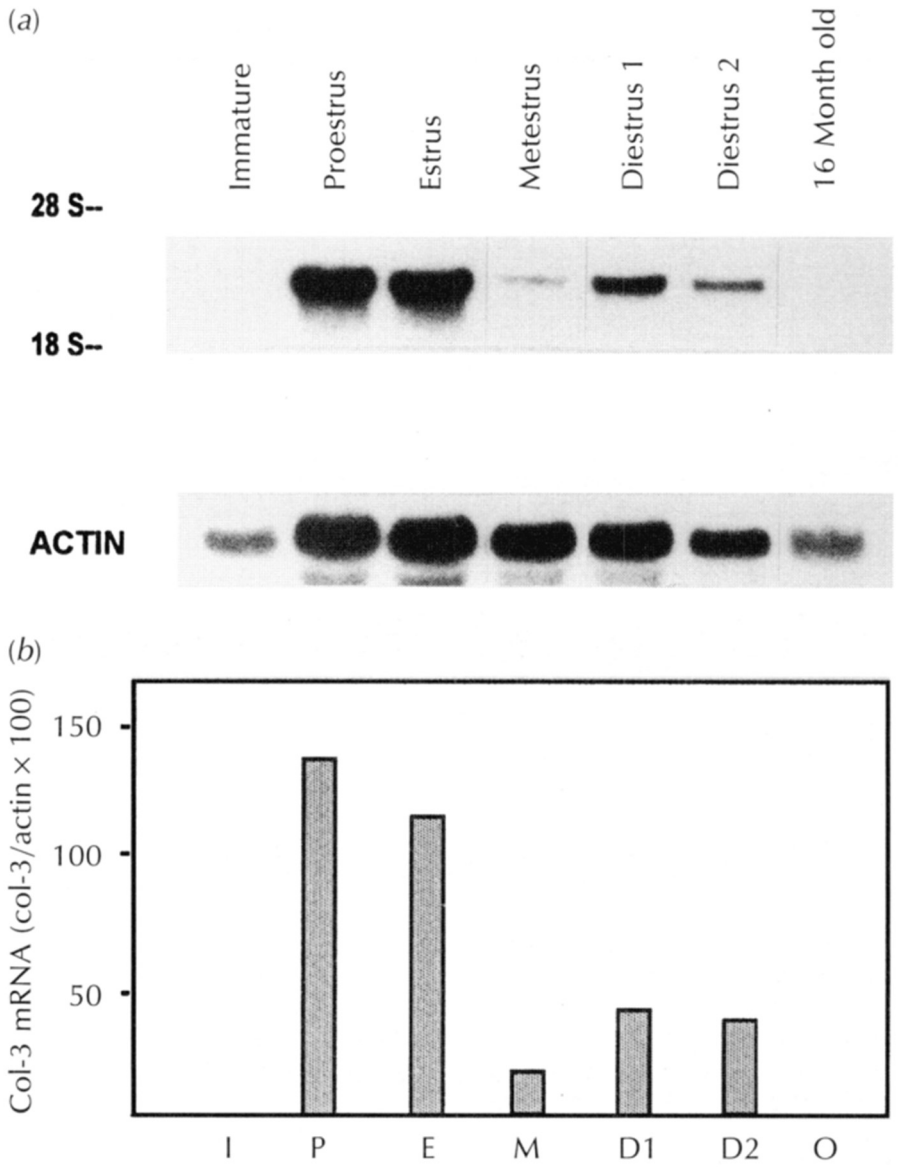

Figure 2 (a) Expression of collagenase-3 (col-3) mRNA in rat ovaries during the different phases of the estrous cycle and in ovaries from immature or old acyclic (persistent estrous) rats. Probes for collagenase-3 and actin were used in Northern blot. Length and conditions of exposure to autoradiography were as in Fig. 1. (6) The hybridization signals of collagenase-3 were scanned with a densitometer and normalized to the amount of total RNA as determined from the hybridization signal obtained with the actin probe. The experiment was replicated three times and the quantification represents the ratio of collagenase-3 to actin signals In a representative experiment.

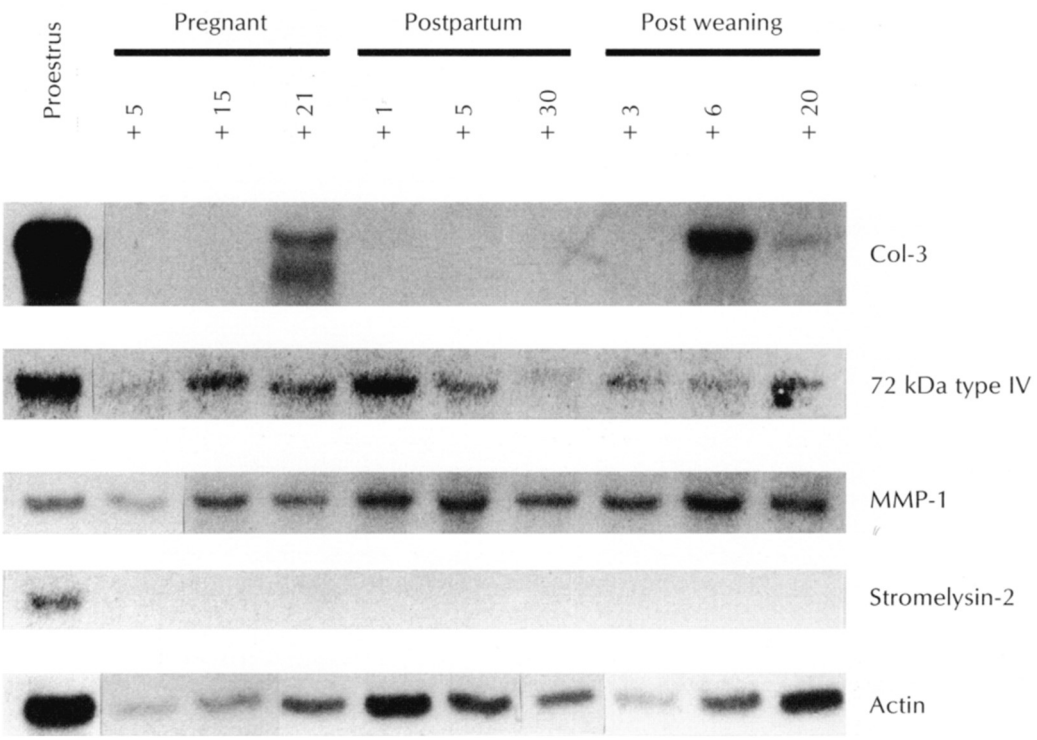

Figure 3 Northern blot analysis of rat mRNA from ovaries taken on days 5, 15 and 21 of pregnancy, 1, 5 and 30 postpartum and 3, 6 and 20 after weaning. In all cases, rats were weaned 9 days postpartum. Samples corresponding to 3 and 6 days after weaning were taken from estrous rats whereas the sample corresponding to 20 days after weaning was from a diestrous rat. RNA from an ovary at proestrus was also analyzed. Filter was sequentially hybridized with probes corresponding to collagenase-3 (col-3), 72 kDa type IV collagenase, human interstitial collagenase (MMP-1), stromelysin-2 and actin. Length and conditions of exposure to autoradiography were as in Fig. 1, with the exception that filters hybridized with 72 kDa type IV collagenase and MMP-1 were exposed for two weeks.

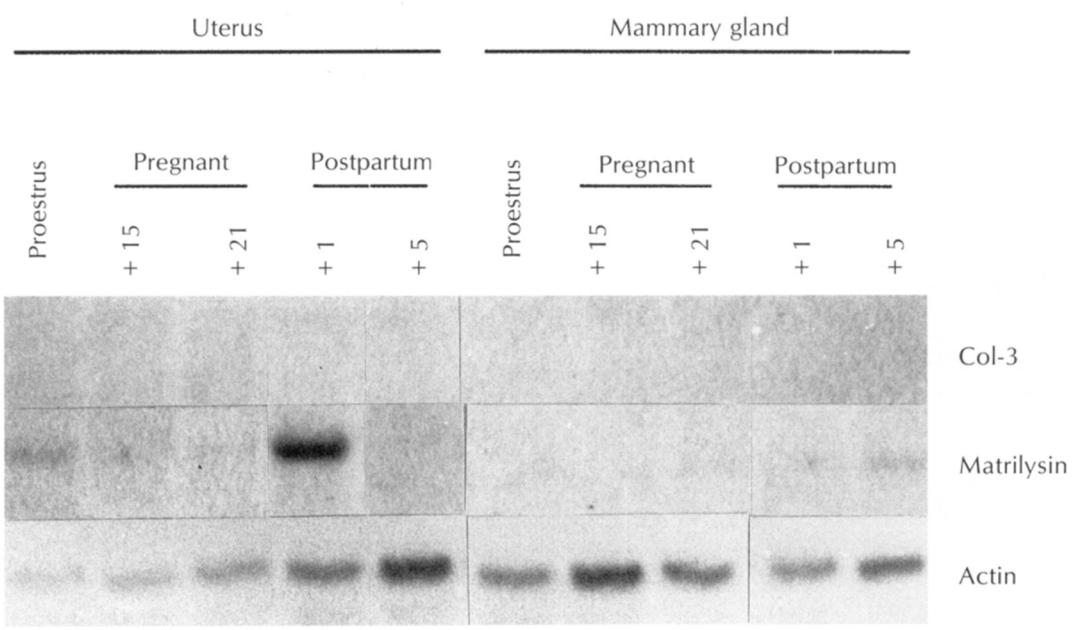

Figure 4 Expression of collagenase-3 mRNA in rat uterus and mammary gland. Probes for collagenase-3 (col-3) and matrilysin were used in Northern blot analysis. Filters were exposed to autoradiography at — 70 °C for 7 days. Hybridization with an actin probe was performed to verify equal sample loading.

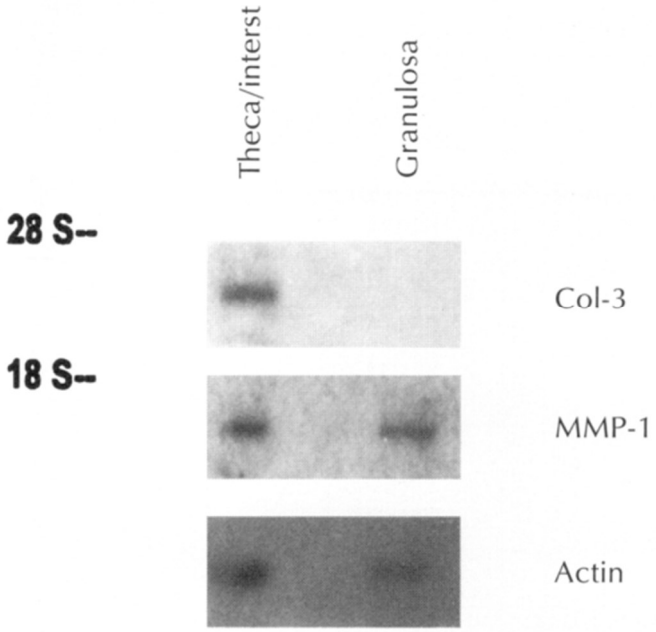

Figure 5 Granulosa and residual tissue expression of collagenase-3 mRNA. Granulosa cells and residual tissue (theca/interst) were obtained as described in the Materials and Methods section. Total RNA (10 µg) was analyzed by Northern blot and sequentially hybridized with probes corresponding to collagenase-3 (col-3), human interstitial collagenase (MMP-1) and actin. Length and conditions of exposure to autoradiography were as in Fig. 3.

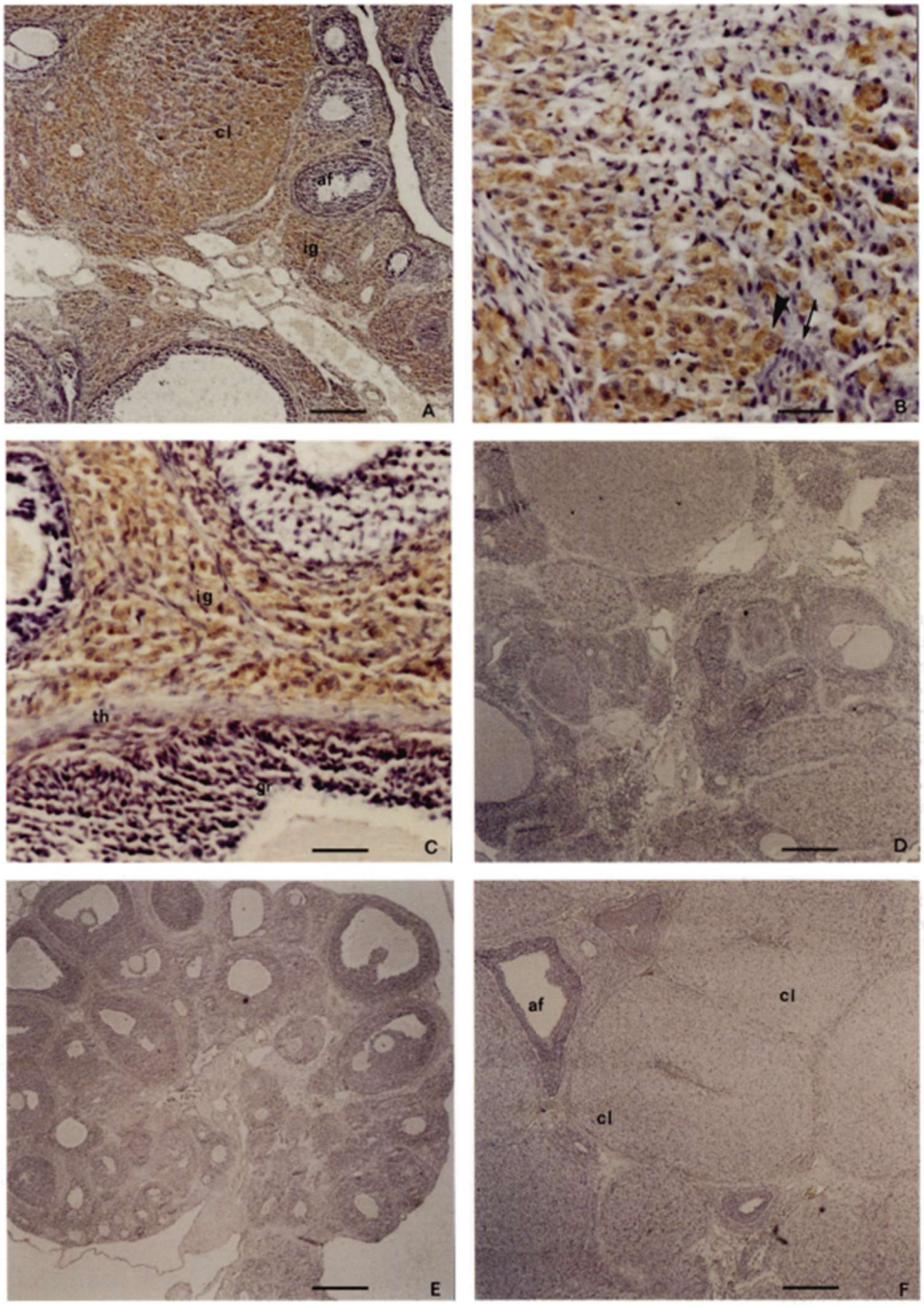

Figure 6 Immunohistochemical localization of collagenase-3 in rat ovary. Ovaries were collected from adult rats on the morning of the day of estrus (A,B,C) and on day 15 of pregnancy (D). Immunoreactivity of collagenase-3 was observed in the theca cells (th) of the antral follicles (af), in the interstitial gland cells (ig) and in luteinized granulosa cells (arrowheads) from the corpota lutea (cl). No significant Immunoreactivity was observed in granulosa cells (gr) or luteinized theca cells (arrows). Ovaries from pregnant rats (D) were negative for the Immunostaining. (E, F) Ovaries from 26-day-old immature rats, (E) before ot (F) 48 h after stimulation with PMSG and hCG (see Materials and Methods). Experimental induction of ovulation was verified by corpus luteum formation 48 h after treatment (F). Immunoreactivity was negative in both cases. Tissue sections were Incubated with anti-collagenase-3 diluted 1:500, and counterstained with hematoxylin.

In A,D,E,F the scale bar=200 μ ; In B,C the scale bar=40 μ . A minimum of ten different ovaries were examined and in each case at least five different sections were subjected to immunostaining with anti-collagenase-3 antibodies.

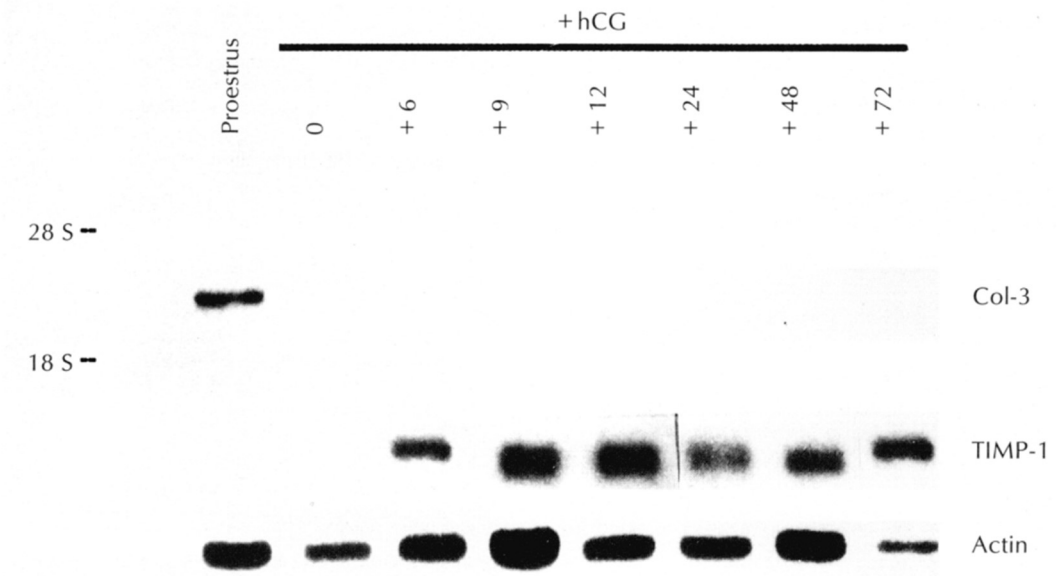

Figure 7 Northern blot analysis of ovarian RNA of immature rats after hCG stimulation. Rat ovaries were collected at the time of hCG administration (0 h) and at 6, 9, 12, 24, 48 and 72 h after hCG injection. RNA from the proestrous rat ovary was also analyzed as a positive control of collagenase-3 expression. Filter was hybridized with collagenase-3 (col-3) probe and thereafter with a probe for TIMP-1 and actin. The experiment was replicated four times. The figure shows the results of a representative experiment.