Quantitative and cytochemical studies of mast cell proteases in rat ovaries and uteri in various reproductive states

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A role for mast cell proteases (RMCP I and II) in the cyclical remodelling of ovarian and uterine tissues of rats was investigated in the oestrous and pregnancy cycles using immunocytochemistry and enzyme-linked immunosorbent assays. The concentrations of RMCP I exceeded that of RMCP II by 100-fold in both tissues, but were always much higher in uteri than ovaries. Most of the protease activity in the uterus was located in the myometrium, whereas it was more focally distributed in the hilus and medulla of the ovary. Protease activity was confined to mast cells identified by metachromatic staining and no single cell contained both proteases. The concentrations of RMCP I and II in the two organs did not fluctuate throughout the 4-day oestrous cycle. Neither were RMCP I concentrations in the uterus altered by administration of diethylstilboestrol to ovariectomized animals, although total amounts per uterus were substantially greater than in controls. Concentrations of RMCP I were substantially reduced in the uterus after day 6 of pregnancy and rose during the puerperium. The reduction was greater in pregnant than in pseudopregnant horns and tended to be lower in the vicinity of conceptuses than between them. The physiological significance of the lower mast cell protease concentrations is unclear, although their absence may contribute to the decreased protein catabolism during pregnancy.

Introduction

The role of mast cells in the female reproductive tract and ovary, in which they are widely distributed, has been the subject of investigation and speculation for several decades. The mast cell mediator, histamine, was first implicated in decidualization of the rodent uterus for implantation by Shelesnyak (1960), and later studies suggested that it had a role in ovulation (Kobayashi *et al.*, 1983). Changes in numbers of mast cells occur in reproductive cycles and pregnancy (Iversen 1960; Brandon and Evans, 1983; Krishna *et al.*, 1989; Gaytan *et al.*, 1991) and mast cell degranulation coincides with the preovulatory surge of LH (Jones *et al.*, 1980; Krishna and Terranova, 1985). The numbers of granulated mast cells and concentrations of histamine in the uterus fall at the time of implantation (Marcus *et al.*, 1964; Brandon and Bibby, 1979) and a combination of H₁ and H₂ antagonists partially inhibit implantation in rats (Brandon and Wallis, 1977). The physiological significance of histamine in reproduction still remains unclear, however, and the issue is clouded further by the observation in *W* mutant mice (deficient in both mast cells and oocytes) that pregnancies can be established (Loutit *et al.*, 1982; Wordinger *et al.*, 1985; Wordinger

et al., 1986). Much less attention has been paid to the possible roles of either mast cell heparin or monoamines than to histamine in reproduction (McKercher *et al.*, 1973).

In view of the well-established presence of serine proteases in mast cells, representing as much as 30% of the total granule mass, it is surprising that their potential significance for fertility has been completely overlooked so far (Darzynkiewicz and Barnard, 1967). Two subsets of mast cells can be identified according to protease composition in rat tissues. Rat mast cell protease I (RMCP I), a highly cationic and insoluble enzyme, is mainly located in mast cells of connective tissue. In contrast, RMCP II, a highly soluble and less cationic protease, characterizes mast cells in mucosal tissues (Gibson and Miller, 1986; Gibson *et al.*, 1987). These enzymes are chymases; collagen Type IV in basement membranes is one of their substrates (Sage *et al.*, 1979) and another activity is the activation of procollagenases in fibroblasts (Birkedal-Hansen *et al.*, 1976). There is, therefore, a basis for suspecting that these proteases contribute to remodelling the extracellular matrix of (1) the ovary as follicles and corpora lutea grow and regress or (2) the uterus during the oestrous cycle and pregnancy particularly during *postpartum* involution when the rate of collagen breakdown and collagenase activity are highest (Geyer *et al.*, 1977; Woessner, 1979). We have therefore investigated the distributions and concentrations of mast cell proteases in the female reproductive tract during the 4-day oestrous cycle, pregnancy and puerperium.

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Fig. 1. (a) Section of uterus demonstrating the immunofluorescent localization of rat mast cell protease I (RMCP I) in mast cells within the rat myometrium (arrows). Sections were incubated with rabbit antibody to RMCP I followed by ovine anti-rabbit IgG–fluorescein isothiocyanate (FITC). (b) Section of ovary stained as above, showing RMCP I-containing mast cell within the medulla (arrow). (Bar = 40 μm).

Materials and Methods

Preparation of tissue specimens

The oestrous cycles of virgin Sprague-Dawley rats weighing approximately 200 g were monitored by vaginal lavage and staining with Giemsa. Six animals presenting two consecutive 4-day cycles were killed on each of the days of the cycle (pro-oestrus, oestrus, metoestrus, dioestrus), and their uteri and ovaries were dissected. Another group of 25 animals was mated with males of the same strain and autopsies were done between

days 6 and 21 *post-coitum* (day 1 = day of spermatozoa in vaginal smear). Seven nonpregnant controls were examined at the same time. The rats examined on days 6–8, were given an i.v. injection of 1 ml 1% (w/v) Pontamine Sky Blue dye (Searle, High Wycombe) 15 min before death to locate early implantation sites (Psychoyos, 1960). One of each pair of Fallopian tubes in 13 animals was ligated under sodium pentobarbitone anaesthesia (35 mg kg^{-1} i.p.) two weeks before mating to establish pseudopregnancy conditions in one uterine horn with a pregnancy on the contralateral side. An additional group of eight rats was examined on the second day *post-partum*.

Fig. 2. Variations in amounts of rat mast cell protease (RMCP), (a) RMCP I and (b) RMCP II, in the uterus during the 4-day oestrous cycle. PRO: pro-oestrus; OE: oestrus; MET: metoestrus and DI: dioestrus. The results are expressed as means $\pm$ SEM of RMCP per unit wet weight of tissue $\square$ and per uterus $\square$.

Fig. 3. Variations in amounts of rat mast cell protease (RMCP), (a) RMCP I and (b) RMCP II, in the ovary during the 4-day oestrous cycle. PRO: pro-oestrus; OE: oestrus; MET: metoestrus and DI: dioestrus. The results are expressed as means $\pm$ SEM of RMCP per unit wet weight of tissue $\square$ and per ovary $\square$.

A further group of 16 rats was ovariectomized under the same anaesthetic and treated daily on the fourth to seventh post-operative days, inclusive, with either $1 \mu\text{g}$ or $10 \mu\text{g}$ diethylstilboestrol kg^{-1} (DES) or arachis oil (0.2 ml) s.c. These rats were killed on the following day.

The animals were killed by cervical dislocation and the ovaries and uterine horns were dissected and blotted to remove surface blood and uterine luminal fluid. For the gravid horns, two implants and intervening uterine segments were prepared from each animal. The tissues were weighed and homogenized with a Polytron homogenizer (Northern Media Supply Ltd, North Cave, UK) at dilutions of either 1:5 or 1:20 (w:v) in Tris-HCl buffer (20 mmol l^{-1} , pH 7.5) containing $1.5 \text{ mol NaCl l}^{-1}$ and stored at -20°C . Additional pieces of ovarian and uterine tissue were fixed in 4% paraformaldehyde (pH 7.4) overnight and prepared as $6 \mu\text{m}$ paraffin wax sections on gelatin-subbed slides for immunocytochemistry.

Immunocytochemistry

The locations of RMCP I and RMCP II in tissues were determined by the paired immunofluorescence technique described by Huntley *et al.* (1990). Briefly, tissue sections were pretreated with 3-amino-9-ethyl-carbazole to block nonspecific

Fig. 4. The effects of administering diethylstilboestrol to ovariectomized rats on $\square$ uterine weight, $\square$ RMCP I expressed in $\mu\text{g g}^{-1}$ uterus, and $\blacksquare$ RMCP I in μg per whole organ. The means and SEMs are given.

fluorescence in eosinophils and then incubated sequentially with rabbit F(ab')₂ anti-RMCP I ($10 \mu\text{g ml}^{-1}$), ovine anti-rabbit IgG-methylrhodamine isothiocyanate (TRITC) (1/50) (Cooper Biochemicals, Philadelphia), monoclonal anti-RMCP II ($10 \mu\text{g ml}^{-1}$) and ovine anti-mouse IgG-fluorescein isothiocyanate (FITC) (1/100) (Binding Site Ltd, Birmingham). All antibodies were diluted in 10% ovalbumin PBS, and slides were washed six times in PBS between incubation with each reagent. High background autofluorescence of granules in macrophages observed with green light (TRITC) excitation was

Fig. 5. Effects of different reproductive states on mean $\pm$ SEM concentration of RMCP I in the rat uterus. Concentrations in pregnant uteri (P) were much lower than in nonpregnant (NP) ($P < 0.01$) and post-parturient horns (PP) ($P = 0.05$), whereas those in pseudopregnant horns (PSP) were significantly higher than in corresponding pregnant horns ($P < 0.05$). The pregnancy values for days 6–8 (6–8d) and 16 (d16) were lower in uterine tissue immediately adjacent to conceptuses (■) than in intervening segments (▨).

reduced considerably, although not eliminated, by immersing slides in chloroform for 30 min. The mast cell identity of fluorescing cells was confirmed by additional sections reacting with rabbit anti-RMCP I followed by goat anti-rabbit-FITC (Sera-Lab Ltd, Crawley Down). Further sections were also stained with 0.5% toluidine blue (pH 4.5) to compare the tissue distribution of granulated mast cells (Enerback, 1966).

Quantitation of mast cell proteases

The amounts of RMCP I and II in tissue extracts were measured by an enzyme-linked immunosorbent assay (ELISA) technique (Huntley *et al.*, 1990). Dynatech M129B plates (Dynatech Laboratories Ltd, Billingshurst) were coated with either an affinity-purified polyclonal sheep antibody to RMCP I or a murine monoclonal antibody to RMCP II. After incubating at room temperature with known concentrations of standard protease preparations or different dilutions of the test sample, a second antibody was added. In the first case, this was rabbit anti-RMCP I and, in the second, ovine anti-RMCP II labelled with horseradish peroxidase according to the method of Nakane and Kawaoi (1974). A third antibody was added for detecting RMCP I, namely, goat anti-rabbit IgG conjugated to horseradish peroxidase (Sera-Lab). The peroxidase activity of the conjugates was estimated after they were reacted with orthophenylene diamine (400 mg l^{-1}) in $0.1 \text{ mol citrate-phosphate buffer l}^{-1}$ (pH 5.0) containing $0.4 \text{ mg hydrogen peroxide l}^{-1}$ (Sigma, London). The reactions were halted by addition of $2.5 \text{ mol H}_2\text{SO}_4 \text{ l}^{-1}$ and the coloured products were measured using a Titertek Multiscan plate reader (Flow Laboratories, Irvine). Concentrations were compared by ANOVA, Duncan's multiple range test and the paired *t* test.

Results

Tissue distribution of mast cell proteases

Intense red (TRITC) fluorescence for RMCP I was observed in cells of the uterine serosa, mesometrium and myometrium.

These fluorescent cells were generally large ($> 15 \text{ }\mu\text{m}$ diameter), granular in appearance and had a scattered distribution corresponding to toluidine blue-stained mast cells in comparable sections. The density of RMCP I-containing cells in the ovary was lower, but they too had a similar distribution to meta-chromatically stained cells. Most were observed in the hilus, close to the larger ovarian blood vessels or in the medulla; few were found in the cortex. Despite the persistence of some autofluorescence after quenching sections in chloroform, RMCP I fluorescence could be localized unequivocally in mast cells with the antibody-FITC conjugate (Fig. 1a, b).

Few mast cells containing RMCP II were observed in either tissue, a finding which is in accord with the much lower tissue concentrations of RMCP II compared with those for RMCP I (see next section). Few FITC-positive cells were noted in the uterine mucosa, although these fluoresced weakly. Cells exhibiting dual fluorescence for RMCP I and II were not observed in these tissues.

Concentrations of proteases in different reproductive states

Protease concentrations in the ovary and uterus during the oestrous cycle are shown (Figs 2 and 3) which indicate that amounts of RMCP I in both organs exceeded those of RMCP II by 100-fold. The nonpregnant rat uterus contained approximately $10 \text{ }\mu\text{g}$ of RMCP I *in toto*, corresponding to a concentration of $20\text{--}40 \text{ }\mu\text{g g}^{-1}$ (Fig. 2). By contrast, the ovary contained $0.1\text{--}0.2 \text{ }\mu\text{g}$ of RMCP I and only a trace of RMCP II ($0.001 \text{ }\mu\text{g}$) (Fig. 3). Individual organs contained widely variable amounts of protease, but there was no evidence of significant variation according to the stage of the cycle in either organ whether expressed as a total amount or concentration per unit organ weight ($P > 0.05$).

Oestrogen treatment of ovariectomized rats produced the expected dose-dependent uterotrophic response ($P < 0.01$) (Fig. 4). The amounts of RMCP I per uterus rose with dose and at the same rate with uterine weight ($P < 0.05$), whereas protease concentrations in the two treatment groups remained the same as those in controls. The amounts of RMCP II in this experiment and in the pregnancy study followed a similar pattern; they are not recorded here because only trace amounts were present.

Concentrations of RMCP I in the uterus were strikingly lower in pregnancy than in virgin animals ($P < 0.01$) or during the puerperium ($P = 0.05$) (Fig. 5). Concentrations fell abruptly to approximately 20% of the nonpregnant values by days 6–8 of pregnancy (data combined, as there was no difference between these days). RMCP I concentrations appeared to be higher at 16 days of gestation than at earlier stages of pregnancy ($P < 0.01$), although they failed to reach statistical significance when compared with pseudopregnancy or day 21 ($P > 0.05$). By the second day *post partum* RMCP I concentrations had risen appreciably, although they had not reached nonpregnant values ($P < 0.01$).

Concentrations of RMCP I in pseudopregnant uterine horns on days 6–8 were lower than those in nonpregnant controls ($P < 0.01$), but they were significantly higher than the contralateral pregnant horn ($P < 0.05$, Fig. 5). An additional source of variation was the location of specimens taken from pregnant horns. Amounts of protease were significantly lower in the

vicinity of conceptuses than between conceptuses on days 6–8 ($P < 0.005$) and 16 ($P = 0.05$), although not at later stages of pregnancy.

Discussion

Methods for localizing and measuring the two mast cell proteases, RMCP I and II, in rats provide accurate and sensitive monitors of the rat mast cell subpopulations in tissues and organs during physiological and pathological processes (Woodbury *et al.*, 1984; Gibson and Miller, 1986). The present results demonstrate that the major mast cell protease in the rat ovary and uterus is that which normally characterizes connective tissues, namely, RMCP I (Gibson *et al.*, 1987), and the concentrations in the uterus exceeded that in the ovary by about two orders of magnitude.

Tissue concentrations of mast cell proteases are assumed to reflect mainly the amounts of enzyme stored in granules rather than in blood or tissue fluids which contain relatively low concentrations of RMCP I and II (Cummins *et al.*, 1988; J. Huntley, unpublished observations). A physiological role might be inferred if concentrations fell abruptly as a result of degranulation at a particular stage of a reproductive cycle. The absence of significant changes in concentrations or total amounts of either protease during the oestrous cycle suggests, however, that, although they may be acting tonically on the extracellular matrix, they are not responding to hormonal signals and are unlikely therefore to be important components of the mechanism for cyclical remodelling of the ovary and uterus. The substantial variations in concentrations at all stages of the cycle suggest that analysis of mast cell subpopulations may be more rewarding.

The oestrous cycle of Sprague-Dawley rats, normally of only 4 days duration, may be too short for effects of low concentrations of ovarian hormones on long-lived tissue mast cells and their mediators to become apparent. When variations in the density of metachromatically-staining cells in the uterus have been found they have been modest (Gibbons and Chang, 1972). However, even when pharmacological doses of a synthetic oestrogen, diethylstilboestrol, were given for four consecutive days the marked uterotropic responses were not accompanied by effects on RMCP I concentrations. The total amounts of protease were, however, increased at the same rate as tissue wet weight, suggesting that mast cells are being regulated according to tissue mass. Presumably, new mast cells had formed by mitosis of existing cells or by migration and differentiation of stem cells, because there was no immunocytochemical evidence of degranulation. Since RMCPs are thought to be released with histamine, this evidence appears to conflict with studies showing that oestrogen induces degranulation, although the actions of this hormone on mast cells are evidently neither direct nor straightforward (Cocchiara *et al.*, 1992).

If mast cell proteases were involved in remodelling the uterus during the puerperium, it is plausible, in light of evidence of oestrogen-induced degranulation at implantation (Marcus *et al.*, 1964), that the *postpartum* oestrus may trigger their release. The present results do not support this hypothesis, however, as might be anticipated from the aforementioned studies of the oestrous cycle. Rather than decreasing after birth (suggesting

degranulation), concentrations of RMCP I actually rose, confirming that mast cells return to the cell population density of nonpregnancy (Shimizu and Hokano, 1987).

The low concentrations of RMCP I throughout pregnancy were the most striking findings of the whole study and confirm histological evidence that mast cell numbers decline (Hore and Mehrotra, 1988). This change occurred by day 6 and was maintained until the day before birth (day 21) despite a slight upturn at day 16 of pregnancy. This increase appeared to be due to a combination of systemic and local factors. Concentrations of protease in pseudopregnant uterine horns were also substantially lower than in nonpregnant uteri, although it remains unclear whether an ovarian hormone is responsible. On the other hand, they were substantially higher than those of contralateral pregnant horns. There were, furthermore, local variations. The concentrations were lower in the vicinity of conceptuses than between them, suggesting that mechanisms regulating mast cell density in the uterus had been locally suppressed, at least during the early phase after implantation. The absence of mast cells in decidual tissue may be sufficient to explain this difference (J. Brandon, personal communication). Considerable remodelling of connective tissues occurs in the early implantation chamber (Clark *et al.*, 1992), but it is unlikely that the RMCPs make an important contribution to this adaptive process as they remain low in this site throughout pregnancy. On the contrary, the lower activities throughout pregnancy may be contributing to the general lowering of protein breakdown and proteolytic enzymes which is essential for uterine growth to occur (Morton and Goldspink, 1986).

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