Curcumin delays endometriosis development by inhibiting MMP-2 activity

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Endometriosis is a common reproductive disorder believed to be associated with matrix metalloproteinases (MMPs) activities for invasion and remodeling of endometrial tissues. Ectopic endometrium has higher capacity to produce proMMP-2 than eutopic tissues; however, the role of MMP-2 during early phase of endometriosis development is still unclear. In the present study, we investigated the role of MMP-2 in establishment and development of endometriosis in mouse model. The effect of curcumin on regression of endometriosis through protease/antiprotease balance between MMP-2 and TIMP-2 was also examined. After endometrial inoculation into peritoneum, we observed a significant elevation of proMMP-2 activity from day 2 onwards. This increased MMP-2 activity was associated with decreased expression of tissue inhibitor of MMP (TIMP)-2, while a significant up-regulation of active MMP-2 activity was observed from day 3 onwards. The activation of proMMP-2 to active MMP-2 was associated with increased expression of membrane type 1 matrix metalloproteinase (MT1MMP). Curcumin at a dose of 48 mg/kg b.w. repressed the MMP-2 activity via up-regulation of bound TIMP-2 expression, thus delayed endometriosis development. In addition, curcumin inhibited production of active MMP-2 by down-regulating MT1MMP expression. Moreover, endometriotic progression was directly linked with increased MMP-2/TIMP-2 ratio which was delayed by curcumin pretreatment. In summary, our study documents the regulation of MMP-2 activity by TIMP-2 during the early phase of endometriosis development and inhibitory action of curcumin thereon.

Keywords: Curcumin, Endometriosis, Matrix metalloproteinase, Tissue inhibitor of MMPs

Endometriosis is a common benign inflammatory gynaecological disease of reproductive women that leads to infertility. The patho-physiology of the disease is associated with chronic pelvic pain, progressive dysmenorrhoea and dyspareunia due to development of endometrial implants within extra-uterine areas like fallopian tube, ovary and peritoneum. The biochemical mechanism for the development and progression of this disease is still unclear. According to the hypothesis of metaplasia, endometriosis in some cases develops due to sex-hormone-dependent transformation of peritoneal cells into Mullerian-type epithelium. The other and most accepted theory is Sampson’s reflux implantation theory, where retrograde menstruation is believed to deliver endometrial debris to peritoneum and ovary, leading to development of endometriosis. Thus, establishment of endometriosis requires cellular invasion and remodelling of extracellular matrix (ECM).

Matrix metalloproteinases (MMPs), a Zn-dependent endopeptidases play important roles in ECM remodelling both in normal physiology and pathology. In endometriosis, several MMPs including MMP-1, -3, -9 and -11 have shown increased activities in patients than normal healthy women. Especially, increased MMP-9 (gelatinase B) activity has been reported by several researchers for inflammatory responses as well as ECM remodelling along with progression of the disease. During endometriosis, early induction of MMP-3 activity is distinct and involves activation of other MMPs including MMP-9. Although some previous reports have also shown increased MMP-2 activity during endometriosis, the mechanism is not well established and its role in endometriosis development is still under investigation.

The regulation of MMP-2 activity is mainly controlled by its endogenous inhibitor — the tissue inhibitor of metalloproteinase (TIMP)-2, which inhibits the MMP-2 activity by interacting with its

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Abbreviations: ECM, extracellular matrix; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TIMP, tissue inhibitors of MMPs; MT1MMP, membrane type 1 matrix metalloproteinase.

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N-terminal domain. On the other hand, the membrane type 1 matrix metalloproteinase (MT1MMP) is known to activate the 72 kDa progelatinase A after binding to TIMP-2. MMP-2 plays important roles in pathological angiogenesis and imbalance in MMP-2 and TIMP-2 ratio might play critical role in modulating angiogenesis during early development of endometriosis.

Curcumin, a polyphenol isolated from Curcuma longa has shown anti-inflammatory activity against wounds and gastric ulcers. It is reported to decrease reactive oxidative stress and inhibit MMPs. During endometriosis, curcumin pretreatment inhibits inflammatory cytokines and MMP-9 and -3 expression. Curcumin also inhibits cellular proliferation by suppressing cyclooxygenase-2 and causes inhibition of cell cycle at G2 phase and subsequent apoptosis. The promotion of apoptosis by curcumin follows mainly intrinsic apoptotic pathway that accelerates regression of endometriosis. Curcumin has demonstrated potent anti-endometriotic activity, though its effect on MMP-2 and TIMP-2 during early endometriosis is still not well characterized.

In this study, we have investigated the regulation of MMP-2 activity by TIMP-2 during early stage of endometriosis development in mouse model. The effect of curcumin on regression of endometriosis through protease/antiprotease balance between MMP-2 and TIMP-2 has also been studied.

Materials and Methods

Chemicals

Gelatin, Triton X-100, protein A agarose bead, protease inhibitors mixture, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium and purified curcumin (~80% HPLC) were obtained from Sigma Aldrich Inc, St. Louis, MO, USA. Pre-stained protein molecular weight markers were purchased from Fermentas Inc, Washington, DC, USA. Mouse reactive polyclonal anti-MMP-2, anti-TIMP-2, anti-MT1MMP and anti-GAPDH antibodies were obtained from Santa Cruz Biotechnology Inc, California, USA. All other chemicals were purchased from Sisco Research Laboratories, Mumbai, India.

Induction of peritoneal endometriosis in BALB/c mice: Protective studies

Female adult BALB/c mice of 6–8 weeks old, bred in house with free access to food source and water were used in all experiments. Animal experiments were carried out following the guidelines of the Animal Ethics Committee of the Institute.

Induction of peritoneal endometriosis was carried out by method of Somigliana et al. with little modification using ovariectomized mice. Briefly, on day 0 the donor mice were anesthetized (ketamine 12 mg/kg body wt) and sacrificed to obtain uterine horns under sterile conditions. The endometrium was carefully teased out and chopped and suspended in 0.6 ml of sterile phosphate buffer saline (PBS) and inoculated into the peritoneal cavity of recipient mice containing subcutaneous implants of estradiol-17β (25 µg/ml) pellet with a ratio of one donor to two recipients. These uterine tissues of donor mice were used as control (D0). Curcumin was administered once daily at 48 mg/kg b.w. intraperitoneally (i.p.) for three days (day -1, 0 and +1) to test its protective effects against endometriosis. Mice, 4 each in a group, were sacrificed on day 1 (D1), day 2 (D2), day 3 (D3) and day 5 (D5) after induction of endometriosis and endometriotic implants were collected and preserved. All animal experiments were repeated independently for three-times.

Tissue extraction

Tissues were suspended in PBS containing protease inhibitors and minced at 4°C. The suspension was centrifuged at 12,000 g for 15 min and supernatant was collected as PBS extracts. The pellet was further extracted in lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, and protease inhibitors) and centrifuged at 12,000 g for 15 min to obtain Triton X-100 (Tx) extracts. Protein was estimated by Lowry method.

Gelatin zymography

For assay of MMP-2 activity, tissue extracts (30 µg protein/lane) were electrophoresed in 8% SDS-polyacrylamide gel containing 1 mg/ml gelatin under non-reducing conditions. The gels were washed twice in 2.5% Triton X-100 and then incubated in calcium assay buffer (40 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 10 mM CaCl2) for 18 h at 37°C. Gels were stained with 0.1% Coomassie blue, followed by destaining. The zones of gelatinolytic activities appeared as negative staining. Quantification of zymographic bands was done using densitometry linked to proper software (Lab Image, Kapelan GmbH, Leipzig, Germany).

Immunoprecipitation

Tissues were washed with PBS and subsequently lysed in ice-chilled lysis buffer (10 mM Tris-HCl,
pH 8.0, 150 mM NaCl, 1% Triton X-100 and protease inhibitors) by incubation on ice for 45 min. The lysate was centrifuged at 12000 g for 10 min and the supernatant was incubated with primary antibody (rabbit anti-MMP-2 antibodies) for 6 h at 4°C with simultaneous mixing on a rotating wheel and centrifuged at 2000 g for 5 min. The supernatant was further incubated for 12–14 h with activated protein A agarose at 4°C and beads were pelleted down by centrifugation at 13000 g for 5 min at 4°C. Beads were then washed with ice-chilled lysis buffer, followed by PBS. Immunoprecipitated proteins were obtained by treating the beads with sample loading dye and supernatants were subjected to Western blot using goat anti-TIMP-2 antibody.

**Western blot**

Tissue extracts (100 µg/lane) were resolved by 10% reducing SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked for 2 h at room temperature in 3% BSA solution in Tris-buffered saline and tween 20 (TBST) containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.02% Tween 20. Tween 20, followed by overnight incubation at 4°C in 1:200 dilution of the respective primary antibodies in TBST containing 0.2% BSA. The membranes were washed five-times with TBST and then incubated with alkaline phosphatase-conjugated secondary antibody (1:2000) for 1.5 h. The bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate solution.

**Statistical analysis**

Data were fitted using Sigma plot. Data obtained from three independent experiments were represented as the means ± SEM. P<0.05 was accepted as level of significance. The statistical analysis of the data was done using GraphPad Instat 3 software. Comparison between groups was done using One-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test.

**Results**

Curcumin suppressed the upregulation of MMP-2 activity during early phase of endometriosis in mouse model

After inoculation of endometrial fragments into the peritoneal cavity of mice, we first checked MMP-2 activity during early phase of endometriosis by gelatin zymography (Fig. 1A). There were no stable endometrial implants for the first 24 h and no change in MMP-2 activity relative to control (D0) was observed. After 48 h, inclusions were found to be stable at implantation sites and upregulated proMMP-2 activity. We observed a strong correlation between endometriosis progression and the activity of both secreted and synthesized proMMP-2 from 2nd day onwards. With the establishment of stable implantation, proMMP-2 activity was gradually upregulated showing ~2.5-fold increase at day 5 (Fig. 1B, C). No active MMP-2 was observed up to 48 h in the endometrial implants. From day 3, we observed significant upregulation of active MMP-2, showing increased activity by ~2-fold at day 5 (Fig. 1B, C). Pre-treatment with curcumin at a dose of 48 mg/kg b. w. suppressed the upregulation of proMMP-2 activity in a time-dependent manner and delayed the endometriotic development. Curcumin also inhibited the activation of proMMP-2, as no active MMP-2 was observed during early stage of endometriosis development in mouse model (Fig. 1).

Upregulation of TIMP-2 and inhibition of MMP-2 expression during regression of endometriosis by curcumin

To further confirm the role of MMP-2 in early stages endometriosis, both untreated and treated samples were subjected to immnoblottting with mouse reactive MMP-2 antibody. MMP-2 expression was upregulated by ~3.5-fold during early endometriosis, whereas curcumin (48 mg/kg b.w.) pre-treatment showed no significant upregulation of MMP-2 expression (Fig. 2A, B). To address the question whether there was a direct regulation of protease/antiprotease balance with disease development, we checked TIMP-2 expression using Western blot analysis (Fig. 2A). TIMP-2 expression followed a relatively negative correlation with MMP-2 during endometriosis establishment (Fig. 2A, C). From day 2 onward of endometrial implantation, TIMP-2 showed ~50% down-regulation of the expression which gradually decreased up to ~20% with time. Pre-treatment with curcumin upregulated TIMP-2 expression by ~2-fold in time-dependent manner. Thus, when we plotted the expression ratio of MMP-2 and TIMP-2 with duration of implantation (Fig. 2D), we observed increased ratio during endometriosis progression that reached up to ~5-fold. Curcumin treatment attenuated the MMP-2/TIMP-2 ratio little below the control value, indicating inhibition of endometriotic development.

Involvement of MT1MMP and protease bound TIMP-2 in regulation of MMP-2 activity during endometriosis

To investigate whether the MMP-2 activity was regulated by MT1MMP, we checked MT1MMP
expression by immunoblotting (Fig. 3A). A gradual increased expression of MT1MMP up to ~2-fold was observed with time in early endometriosis with no treatment of curcumin, while pre-treatment with curcumin (48 mg/kg b.w.) down-regulated the MT1MMP expression by ~40% than control sample (Fig. 3A, B). To investigate whether inhibition of TIMP-2 on MMP-2 activity resulted from complex formation, immnprecipitation of MMP-2 was carried out using protein A-conjugated agarose beads and samples were subjected for immunoblotting against TIMP-2 (Fig. 3C, D). The results showed that endometriosis progression was related with decreased complex formation between MMP-2 and TIMP-2. In
Fig. 2—Inhibition of MMP-2 for endometriotic development by curcumin is associated with TIMP-2 [Western blotting as described in ‘Materials and Methods’ was performed to monitor expression of MMP-2 and TIMP-2 using equal amounts (100 µg) of PBS extracts (A) and respective graphical representation (B) and (C). Ratio of MMP-2 and TIMP-2 expression was represented in histogram (D). Protein band intensities were quantified by densitometric analysis using Lab image software from three blots from independent experiments in each case. Values are ± SEM. Sample number n = 12. ***p<0.001; **p<0.01; *p<0.05 and NS, non-significant vs appropriate control]

Fig. 3—MT1MMP and bound TIMP-2 regulates MMP-2 activity during early endometriosis [Western blot was performed with curcumin treated and untreated endometriosis using equal amount of protein (100 µg) with Tx extract (A) and corresponding graphical representation (B). MMP-2 was immunoprecipitated as described in ‘Material and Methods’ and bound TIMP-2 was detected by immnoblotting using goat anti-TIMP polyclonal antibody (C) and expression represented in histogram (D). Protein band intensities were quantified by densitometric analysis using Lab image software from three blots from independent experiments in each case. Values are ± SEM. ***p<0.001; **p<0.01; *p<0.05 and NS, non-significant vs appropriate control]
contrast, curcumin-treated samples of day 3 showed significantly increased complex formation between TIMP-2 and MMP-2, resulting in inhibition of MMP-2 activity as well as endometriosis development (Fig. 3C, D).

Discussion

Development of endometriosis in reproductive female is a prolonged process that requires growth factors and oestrogen support. In very early stage of development, endometriosis is quite undetectable; therefore, limited work has been reported so far about role of MMPs in developmental stage of endometriosis. In the present study, we developed a mouse model of endometriosis that mimics endometriosis with support of oestrogen for limited period of time.

Herein, we observed an increased MMP-2 activity during early stages of endometriosis development. Earlier, similar results were reported by Lu et al., where they implanted endometrial fragments from women with and without endometriosis into peritoneum of immune compromised rat and reported increased MMP-2 expression in endometriotic implants. This elevated MMP-2 activity was important for collagen IV degradation in basement membrane and endometriosis development. In our study, increased MMP-2 expression was observed from 2nd day of endometriosis induction, which might be related with increased infiltrated neutrophils and activated macrophages from early stage of endometriosis as reported by Lin et al. Therefore, in addition to scavenging damaged cells, neutrophils and macrophages might be important for MMP-2 secretion. The induction of MMP-2, in turn, might support formation of new blood vessels for endometriotic progression.

The regulation of MMP-2 activity by TIMP-2 is well established; TIMP-2 inhibits MMP-2 activity by interacting with its N-terminal domain. On the other hand, when N-terminal domain of TIMP-2 is preoccupied with the insoluble MT1MMP, C-terminal domain interacts with proMMP-2, forming MT1MMP-TIMP-2-proMMP-2 complex. Another neighbouring MT1MMP, free from TIMP-2 acts as an activator on proMMP-2 complex and carry out the activation. Therefore, availability of TIMP-2 and MT1MMP is very crucial for activation or inhibition of MMP-2 activity. In our study, we observed endometriosis progression was associated with increased MT1MMP with MMP-2 activity, though MMP-2-bound TIMP-2 was limited. On the other hand, curcumin treatment inhibited both MT1MMP and MMP-2 expression, but increased the bound TIMP-2 level.

This finding explained that limited TIMP-2 in the system had the access to act with MT1MMP initiating activation of active MMP-2; but in the presence of excess TIMP-2 and down-regulated MT1MMP expression, no free MT1MMP was available to initiate progelatinase A activation. Rather TIMP-2 bound with MMP-2 caused direct inhibition of MMP-2 activity through interacting by its N-terminal inhibitory domain. In contrast to previous study by Chung et al. that analysed mRNA of MMP-2 and MT1MMP in endometriosis, similar increased expression levels were observed in patient samples. Additionally, MT1MMP is essential for neovessel formation, because it stimulates angiogenesis in vivo by upregulating VEGF-A gene expression. Moreover, we observed increased MMP-2/TIMP-2 ratio during endometriosis progression, which reflected decreased availability of TIMP-2. Curcumin treatment increased the availability of TIMP-2 towards MMP-2, which might result complex formation with curcumin and delays development of endometriosis. Earlier study has also suggested that curcumin causes regression of endometriosis by decreasing inflammatory cytokines, oxidative stress and MMP-9 activity that are associated with decreased cellular proliferation. Additionally, curcumin may induce mitochondrial apoptosis that plays an important role for regression of endometriosis.

In conclusion, our study demonstrated the gradual up-regulation of MMP-2 activity in both pro- and active enzyme levels in early developmental stages of peritoneal endometriosis in mice. This increased activity was associated with increased MT1MMP and limited bound TIMP-2 expression. Curcumin treatment resulted in delayed endometriosis progression by decreasing MMP-2 activity. The inhibition of MMP-2 activity might be due to enhanced binding of MMP-2 with TIMP-2 during regression of endometriosis by the curcumin.

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References