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Curcumin arrests endometriosis by downregulation of matrix metalloproteinase-9 activity

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Curcumin, a polyphenol derived from turmeric (*Curcuma longa)* possesses diverse pharmacological properties including antioxidant, anti-inflammatory and antiproliferative activities. Endometriosis is a gyneocological disorder characterized by growth of endometrial tissues outside uterus that involves aberrant matrix remodeling. In this study the effect of curcumin was studied on surgically developed endometriosis in mice. Endometriosis with varying severity was developed in mice by peritoneal implantation of uterine fragments. The changes in matrix metalloproteinase (MMP)-9 and tissue inhibitor of metalloprotease (TIMP)-1 were investigated in endometriotic tissues following curcumin pre- and post-treatment. Results showed that MMP-9 activity increased gradually in endometriotic tissues with severity and curcumin treatment reversed the MMP-9 activity near to control value. Curcumin administered either post- or pre-endometriosis arrested endometriosis in a dose-dependent manner. It inhibited both MMP-9 activity and its expression at the level of secretion, during regression of endometriotic lesion. In addition, the attenuated activity of MMP-9 was associated with decreased expression of tumor necrosis factor-α (TNF-α) during healing, suggesting the anti-inflammatory property of curcumin. Moreover, curcumin pretreatment prevented lipid peroxidation and protein oxidation in endometriotic tissues. We reported here for the first time the anti-endometriotic property of curcumin via MMP-9 dependent pathway that may lead to new therapeutic intervention.

Keywords: Curcumin, Endometriosis, Matrix metalloproteinase, Tissue inhibitor of metalloproteinase, Tumor necrosis factor-α, Extracellular matrix

Endometriosis, a common gynaecological disorder that affects women of childbearing age is a benign but aggressive disease[1]. The pathophysiology of this disease still remains elusive. The presence of endometrial gland and stroma in the peritoneal cavity rather outside uterus has been suggested to be the cause of endometriosis[2]. For ectopic implantation and growth to occur, degradation of extracellular matrix (ECM) is the primary step that leads to formation of new vasculature[3]. Degradation of ECM occurs by the action of plasminogen, cathepsin D and matrix metalloproteinases (MMPs)[4]. ECM molecules include collagen, structural glycoprotein, fibronectin, laminin, fibrinogen and proteoglycans that constitute a well organized structure surrounding cells[5]. Tissue remodeling involving ECM is mostly regulated by combined action of MMPs and tissue inhibitor of metalloproteases (TIMPs). An imbalance in the expression of MMPs and TIMPs is associated with numerous diseases like tumorigenesis, metastasis, atherosclerosis, arthritis and endometriosis[6].

The MMPs are proteolytic enzymes and various members of this family are overexpressed during pathophysiological conditions[7,8]. They play important role in tumor invasion and metastasis by degrading ECM components. Several MMPs have been implicated in the development of endometriosis[9,10]. Ectopic endometrium is found to express higher level of MMP-9 than the eutopic endometrium in patients with endometriosis[11]. In an *in vitro* cell culture system, high MMP-3 activity has been detected in cultured medium in uterine endometrial cells obtained from patients with endometriosis[12]. In addition, MMP-7 promoter polymorphism is shown to be associated with endometriosis[13]. Besides, imbalance in the ratio of MMPs and TIMPs plays important role in progression of endometriosis[9].

Curcumin (diferuloylmethane), a polyphenol isolated from Indian spice turmeric (*Curcuma longa*) is widely used as food colouring ingredient in curry[14]. The use of rhizome of turmeric as a 'folklore' medicine is known for long. It has traditionally been

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described as an anti-inflammatory agent to treat gastric and intestinal diseases, wound healing and skin diseases. It offers protection against different diseases by inhibition of lipid peroxidation and protein carbonyl formation[15]. It has been shown to scavenge reactive oxygen species (ROS), downregulate cyclo-oxygeanes-2 and Bcl-2 expression, suppress growth factor signaling pathways and inhibit phosphoinositide-3-kinase/AKT pathways[16]. The beneficial effect of curcumin on various diseases may be due to its antioxidant, anti-inflammatory and NF-kβ inhibition properties. MMP-9 downregulation by curcumin is known to associate with alleviation of invasion and metastasis[14-17]. Curcumin also acts therapeutically in gastric, intestinal and liver diseases[18,19]. However, the optimum dose of curcumin for treatment of given diseases is unanswered.

In view of the above findings, curcumin is anticipated to exert protective effect on endometriosis. Also, whether it can alleviate endometriosis in laboratory animals or humans is not known. Thus, in the present study, the effect of curcumin on regression of endometriotic lesions has been investigated in mice. We have examined whether curcumin mediates its effect through modulation of MMPs activity. Overall curcumin ingestion is safe in humans and dose level toxicity in human trials is 8 g/day[15]. Therefore, biological safety along with cost-effectiveness justifies its use for disease prevention.

Here, the activity and regulation of MMPs, especially MMP-9 during disease progression and regression have been investigated in mice. The anti-endometriotic activity of curcumin has been analyzed in endometriotic tissues, following curcumin treatment either pre- or post-administration in surgically developed peritoneal endometriosis in mice. The study suggests that curcumin arrests endometriosis through inhibition of MMP-9 expression and activity.

Materials and Methods

Chemicals

Gelatin from porcine skin, Triton X-100, protease inhibitors mixture, and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium were obtained from Sigma, St. Louis, MO, USA. Mouse reactive polyclonal anti-MMP-9, polyclonal anti-TIMP-1, polyclonal anti-TNF-α and monoclonal anti-β actin antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. All other chemicals were purchased from Genei, Bangalore, India and Sisco Research Laboratory, Mumbai, India.

Animals

Female Balb/c mice of 6–8 wks old, bred in house with free access to food and water were used in all experiments. Experiments were designed to minimize animal suffering and to use the minimum number associated with valid statistical evaluation. Animals were anaesthetized by ketamine (12 mg/kg b.w.) and sacrificed by cervical dislocation. The experiments were carried out, following the guidelines of the Animal Ethics Committee of the Institute. Animals of both control and experimental groups were kept separately under standard controlled conditions.

Induction of peritoneal endometriosis in mice and its protection by curcumin

Induction of peritoneal endometriosis in Balb/c mice was performed using a modified method of the Somigliana *et al*[20]. Briefly, on day 0, the donor mice were anaesthetized by ketamine (12 mg/kg b.w.) and sacrificed by cervical dislocation. Using a sterile technique, the uterine horns were removed and placed in a petridish containing phosphate buffered saline (PBS). After removing the muscle layers, the endometrium was finely chopped using a sharp razor blade. Endometrial fragments thus obtained were suspended in 0.6 mL of sterile PBS and inoculated into the peritoneal cavity of recipient mice with a ratio of one donor to two recipients. Mice inoculated with sterile PBS containing no endometrial fragments were kept in animal house and were used as control. Mice containing five in each groups were sacrificed on 7th (Endo 7), 15th (Endo 15) and 21st day (Endo 21) of post-induction of endometriosis. Uterine tissue of control mice (n = 5) sacrificed on the day of endometriosis induction (Endo 0) was used as control. Different doses of curcumin (16, 32 and 48 mg/kg b.w.) were administered i.p. 30 min prior to administration of endometrial extract to three different groups of mice (n = 5 per group) and once daily for the next 3 days to test their protective effect in endometriosis.

Therapeutic model of peritoneal endometriosis in mice

Mice were induced with peritoneal endometriosis as described above and administered with curcumin (48 mg/kg b.w.) as well as vehicle intraperitoneally, once daily for the following 10 (n = 5 per group) and

20 (n = 5 per group) days from day 15 post-endometriosis. Regression of endometriotic lesions in curcumin-treated and vehicle-treated mice (n = 5) was monitored after sacrifycing them on day 10 and 20 respectively.

Tissue extraction

The endometriotic tissues were suspended in PBS containing protease inhibitors, minced and incubated for 10 min 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 Triton-X 100 containing lysis buffer (10 m*M* Tris-HCl, *p*H 8.0, 150 m*M* NaCl, 1% Triton X-100, and protease inhibitors) and centrifuged at 12,000 *g* for 15 min to obtain Triton X-100 (TX) extracts. Both PBS and TX extracts were preserved at −80°C for future studies[15].

Gelatin zymography

For assay of MMP-9 activity, tissue extracts were electrophoresed in a 8% SDS-polyacrylamide gel containing 1 mg/mL gelatin, under non-reducing conditions. For zymography, 70 µg protein from each tissue extract was loaded equally in all lanes. The gels were washed twice in 2.5% Triton X-100 and incubated in calcium assay buffer (40 m*M* Tris-HCl, *p*H 7.4, 0.2 *M* NaCl, 10 m*M* $CaCl_2$) for 18 h at 37°C[15]. Gels were then stained with 0.1% Coomassie blue, followed by destaining. The zones of gelatinolytic activities appeared as negative staining. Quantification of zymographic bands was performed using densitometry linked to proper software (Lab Image, Kapelan Gmbh, Germany).

Western blotting

Tissue extracts (80 µg/lane) were resolved by 8% 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 20 m*M* Tris–HCl, *p*H 7.4 containing 150 m*M* NaCl and 0.02% Tween 20 (TBST), 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[15]. The blots shown in this study were representative replicates selected from at least three experiments.

Measurement of lipid peroxidation (LPO)

The cytosolic and microsomal fractions of tissue homogenate obtained from peritoneal endometriosis in mice were used for measurement of lipid peroxide content as thiobarbituric acid-reactive species (TBARS). Briefly, 1 mL of each fraction was allowed to react with 2 mL of TCA-TBA-HCl (15% TCA, 0.375% TBA, 0.25 N HCl) reagent, heated in a boiling waterbath for 15 min, cooled and centrifuged. The absorbance of the supernatant was measured for nanomoles of TBARS at 535 nm ($e = 1.5 \times 10^5$ *M*/cm)[15].

Measurement of protein carbonyl content

Protein oxidation was measured as carbonyl content in the low speed supernatant of mouse endometriotic tissues. The tissues were homogenized in 50 m*M* sodium phosphate buffer, *p*H 7.4 in a Potter-Elvehjem glass homogenizer for 2 min to obtain 20% homogenate. After centrifugation at 600 *g* for 10 min, the proteins from 1.0 mL of the supernatant were precipitated with 10% trichloroacetic acid (TCA) and allowed to react with 0.5 mL of 10 m*M* 2, 4-dinitrophenylhydrazine for 1 h. After precipitation with 20% TCA, the protein was washed thrice with a mixture of ethanol-ethyl acetate (1:1), dissolved in 1.0 mL of a solution containing 6 *M* guanidine HCl in 20 m*M* potassium phosphate adjusted to *p*H 2.3 with trifluroacetic acid, centrifuged, and the supernatant was read for carbonyl content at 362 nm (e = 22,000 *M*/cm)[20].

Statistical analysis

Data for the activity and expression of MMP-9 and expressions of TIMP-1and TNF-α were fitted using Sigma plot and represented as the mean values ± S.E.M. $P < 0.05$ was accepted as level of significance. The statistical analysis of the data was carried out using GraphPad Instat 3 software (San Diego, CA, USA). Comparison between groups was carried out using One-way Analysis-of-Variance (ANOVA), followed by Student-Newman-Keuls test.

Results and Discussion

Time-dependent increase in secreted MMP-9 in mouse endometriotic tissues

We first examined the activity of MMP-9 in PBS and TX extracts of endometriotic tissues obtained

from mice under varying severity of endometriosis. The preparation of endometriotic tissue is described in Fig. 1 flow diagram. Gelatin zymography was performed to assess the activity of MMP-9 at the level of secretion and synthesis. Fig. 2A shows that lesion volume in endometriotic tissues increased with duration of the disease. The results showed a strong correlation between lesion volume and MMP-9 activity which was elevated by ~9-fold and ~14-fold at the level of secretion (Fig. 2A) and synthesis (Fig. 2B) respectively, on day 21 post-endometriosis. Data suggested that MMP-9 activity was upregulated with the severity and duration of endometriosis in mouse model. The pretreatment of curcumin at a dose of 48 mg/Kg b. w. suppressed the time-dependent increase in the volume of endometriotic lesions (Fig. 2C, D) demonstrating its anti-endometriotic property.

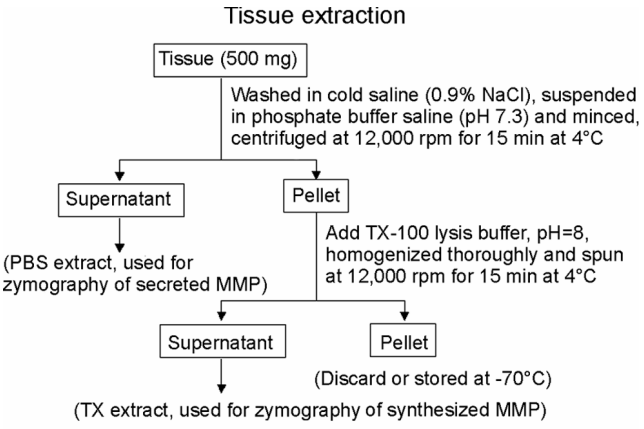

Fig. 1—Preparation of endometriotic tissue homogenate for MMP assay [Endometriotic tissues were isolated from surgically developed endometriosis in mice as described in 'Materials and Methods'. The extraction of inter- and intracellular protein was performed according to the flow chart. The PBS extract was marked as secreted and TX extract as synthesized proteins in all the experiments]

Preventive role of curcumin on endometriosis

As MMP-9 activity was upregulated with the severity of endometriosis and curcumin caused regression of the disease, the role of curcumin on regulation of MMP-9 activity was evaluated. The activity of MMP-9 was tested in control, endometriotic and curcumin pre-treated tissues obtained from mouse model. MMP-9 activity, both at the level of secretion (Fig. 3A) and synthesis (Fig. 3B) decreased significantly in curcumin pre-treated, as compared to untreated endometriotic tissues of day 15. Curcumin at a dose of 16, 32 and 48 mg/kg b. w. showed a gradual decrease in secreted MMP-9 activity by 50%, 70% and 80%, respectively (Fig. 3C). Similarly, synthesized MMP-9 activity decreased by 60%, 70% and 90% using 16, 32 and 48 mg/kg b. w. curcumin respectively (Fig. 3D). Thus, Fig. 3 depicts preventive role of curcumin on endometriosis in a dose-dependent manner.

Therapeutic role of curcumin on endometriosis

To test whether curcumin had any therapeutic effect on endometriosis, we first developed endometriosis and administered curcumin thereafter at a dose of 48 mg/kg b. w. for different durations. It was observed that curcumin significantly suppressed the activity of MMP-9 in endometriotic tissues at the level of secretion and synthesis in a time-dependent

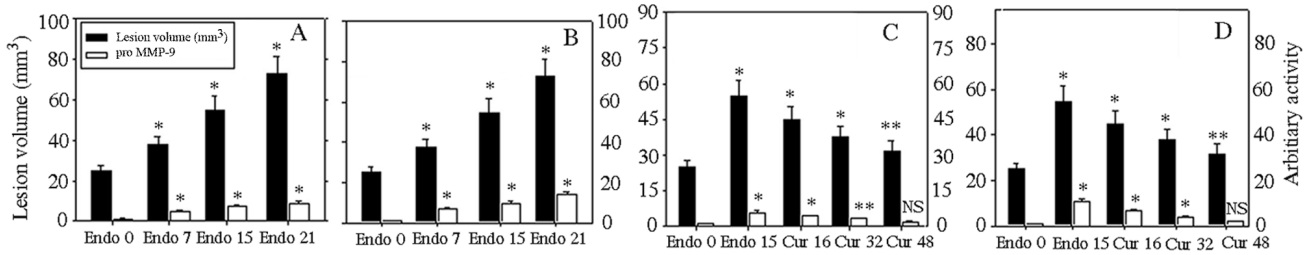

Fig. 2—Relationship of endometriotic lesion volume to secreted and synthesized proMMP-9 activity and the effects of curcumin thereon [Endometriosis was induced over different time periods in mice as described in 'Materials and Methods'. The volume of lesion was monitored on day 0, 7, 15 and 21 endometriosis as well as pretreatment with curcumin, followed by endometriosis induction. Gelatin zymography was conducted to monitor the activity of secreted (**A**) and synthesized proMMP-9 (**B**), as mentioned in Fig. 1 using PBS and TX extracts of control, endometriotic and curcumin-pre-treated endometriosis induced tissues of mice. The lesion volume on particular time points with the corresponding secreted (**A**) and synthesized proMMP-9 (**B**) activity of endometriotic tissues on identical time points was plotted against time and represented as a histogram. Histographic representation of activity values of secreted (**C**) and synthesized proMMP-9 (**D**) with volume of endometriotic lesions against doses of curcumin. Values represented above are ± S.E.M. from three independent experiments. *$P < 0.001$, **$P < 0.01$ and NS, non-significant vs the appropriate control using ANOVA, followed by Student-Newman-Keuls test]

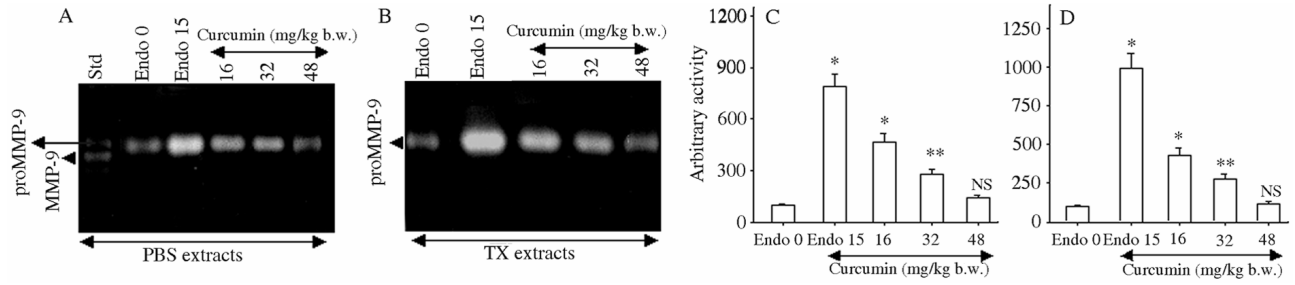

Fig. 3—Preventive role of curcumin on endometriosis and associated proMMP-9 activity [The animals were pretreated with curcumin (16, 32 and 48 mg/kg b.w.) on Endo 15 mice. The animals were sacrificed and tissues were processed as described in the text. Gelatin zymography was conducted to monitor the activity of secreted (**A**) and synthesized proMMP-9 (**B**), as mentioned in 'Materials and Methods' using PBS and TX extracts of control, endometriotic and curcumin-pretreated endometriosis induced tissues of mice. Histographic representation of activity values of secreted (**C**) and synthesized proMMP-9 (**D**) against doses of curcumin as measured by Lab Image software from the above zymograms and three other representative zymograms from independent experiments in each case. Values are ± S.E.M. of the above (**A**) and (**B**) gelatin zymogram and three other representative gelatin zymograms from independent experiments. Sample number n = 40. **P*< 0.001, ***P*< 0.01 and NS, non-significant versus the appropriate control using ANOVA, followed by Student-Newman-Keuls test]

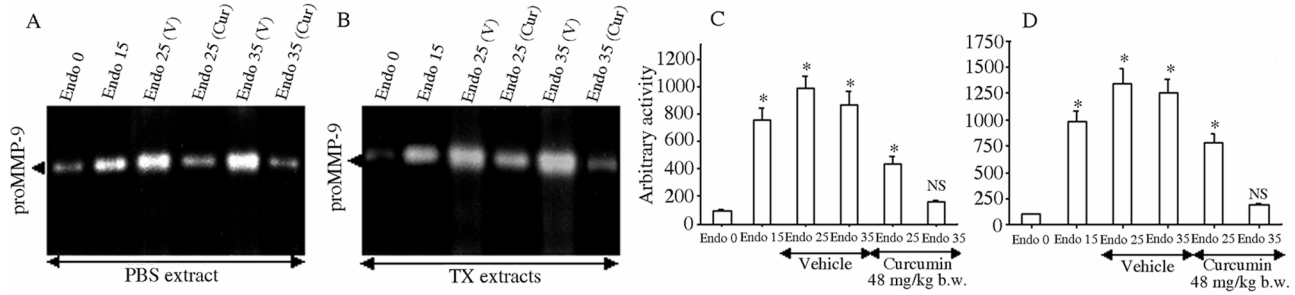

Fig. 4—Therapeutic role of curcumin on endometriosis and associated proMMP-9 activity [The animals were administered intraperitoneally with curcumin (48 mg/kg b.w.) 15th day of post-endometriosis for another extra 10 and 20 days. They were sacrificed on day 10 and 20 post-curcumin or vehicle treatment and gelatin zymography was performed as described in 'Materials and Methods' to detect the activity of secreted (**A**) and synthesized proMMP-9 (**B**). Histographic representation of secreted (**C**) and synthesized proMMP-9 (**D**) activity against time for therapeutic potential of curcumin, as measured by Lab Image software from the above zymograms and three other representative zymograms from independent experiments in each case. Sample number n = 40. Error bars = ± S.E.M. **P* < 0.001 and NS, non-significant versus Endo 0 using ANOVA, followed by Student-Newman-Keuls test]

manner. Curcumin decreased the secreted MMP-9 activity by 45% and 85% on day 10 and 20 post-endometriosis induction (Fig. 4A, C). Similarly, it also inhibited synthesized MMP-9 activity in mouse endometriotic tissues by 50% and 90% on day 10 and 20 post-treatment (Fig. 4B, D). Thus, both preventive and therapeutic studies revealed the anti-inflammatory role of curcumin in regression of endometriosis. We hypothesized that anti-inflammatory role of curcumin might partially, if not solely was involved in regression of endometriosis. On the other hand, curcumin regulated MMP-9 activity through its anti-inflammatory property, while arresting endometriosis.

Regulation of MMP-9 activity via modulation of TNF-α and TIMP-1 expression

Curcumin is reported to regulate inflammatory molecules through the activation of transcription factor NFκβ[15]. It also has an inhibitory effect on pro-inflammatory cytokines in several diseases, including gastric ulcer, breast cancer and arthritis[15, 22-25]. In view of these findings, we investigated the expression level of TNF-α (a pro-inflammatory molecule) in endometriotic tissues and the effect of curcumin thereon. Because MMP-9 activity is coupled to TIMP-1 (endogenous inhibitor of MMP-9) expression[5], TIMP-1 expression in control, endometriotic and curcumin pre-treated endometriotic tissues was also investigated. Western blot was performed to follow the expression pattern of MMP-9, TNF-α and TIMP-1, while β actin was used to confirm equal protein loading in the blots. Results in the present study suggested that curcumin protected against endometriosis through inhibition of TNF-α and elevation of TIMP-1 (Fig. 5). Thus it appeared

Table 1—Effect of curcumin on protein carbonylation and lipid peroxidation in endometriosis

[Peritoneal endometriosis was induced in mice and protection experiment was carried out pretreatment of curcumin (48 mg/kg b.w.) as described in 'Materials and methods'. Mice were sacrificed on day 15 post-endometriosis. Lipid peroxidation (n = 14) and protein carbonyl content (n = 14) from tissue homogenates were measured as described in the text. The values in parentheses represent the percentage reduction of lipid peroxidation and protein oxidation by curcumin. The percentage reduction was measured using the following formula: (value of Endo 15 – value of curcumin)/value of Endo 15 – value of control) × 100. Results expressed as mean ± S.E.M. *$P < 0.001$ and NS, non-significant vs the appropriate control using ANOVA, followed by Student-Newman-Keuls test]

Samples	Protein carbonylation (nmol/mg protein)	Lipid peroxidation (nmol TBARS/mg protein)	
		Cytosolic	Microsomal
Endo 0	0.18 ± 0.04	1.23 ± 0.29	1.42 ± 0.32
Endo 15	0.25 ± 0.08 *	2.23 ± 0.36 *	2.74 ± 0.46 *
Endo 15 + Curcumin (48 mg/kg b. w.)	0.21 ± 0.04 NS (80%)	1.32 ± 0.39 NS (88%)	1.59 ± 0.45 NS (88%)

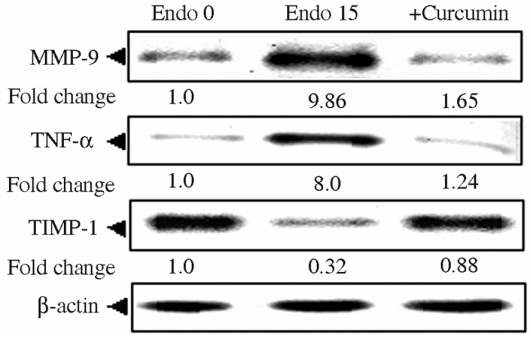

Fig. 5—Regulation of MMP-9 activity via modulation of TNF-α and TIMP-1 expression [PBS extracts of control, endometriotic and curcumin-pretreated endometriotic tissues from mice were subjected to Western blot and probed with polyclonal anti-MMP-9, polyclonal anti-TNF-α, polyclonal anti-TIMP-1 and monoclonal anti-β actin antibodies. Representative Western blots are shown in all cases; and representation of fold changes at protein level for proMMP-9, TNF-α and TIMP-1 as measured by Lab Image software from the above blots and three other representatives blots from independent experiments in all cases. Error bars = ± S.E.M. Sample number, n = 40]

that besides the anti-inflammatory and anti-oxidant role, curcumin served as an anti-endometriotic agent by modulating MMP-9 activity.

Effect of curcumin on protein oxidation and LPO on endometriotic tissues

Curcumin significantly decreased LPO and protein carbonylation in endometriotic tissues. Increased protein oxidation and LPO were observed in endometriotic tissues by 1.5-fold and 2-fold compared to that of control (Table 1). Possibly, the inflammatory signals generated due to endometriosis resulted in excessive oxidative stress in endometriotic tissues. Being a good anti-oxidant, curcumin protected the oxidation of protein and lipid in pre-treated endometriotic tissues. Thus, present study provided further evidence on the anti-oxidant property of curcumin as well as a positive correlation of endometriosis with oxidative stress.

Conclusion

The results of the present study demonstrated that inducible MMP-9 activity in endometriotic tissues enhanced the lesion volume by 3-fold over duration of 21 days. MMP-9 expression had significant effect on the severity of endometriotic lesions and was arrested by curcumin treatment. It inhibited pro-inflammatory cytokines e.g. TNF-α expression during prevention as well as regression of endometriosis. Hence, MMP-9 and TNF-α expression provided a means of evaluating the severity of disease. The aberrant decrease in TIMP-1 expression with the subsequent elevation of MMP-9 aggravates endometriosis[10,11].

Our results provided evidence that curcumin not only attenuated MMP-9 activity and expression, but also upregulated TIMP-1 expression and provided an extra edge in regulating MMP-9 activity. Thus, curcumin potentially regulated MMP-9 activity that was further controlled by TIMP-1 and resulted in healing of endometriosis. Moreover, stabilization of endometriosis requires angiogenesis and curcumin might act as an antiangiogenic agent while regressing endometriotic lesions[26].

The data provided in study might have important clinical implication, since an excess of MMP-9 could lead to aberrant matrix remodeling in peritoneal endometriotic tissues and those could be potentially reversed by curcumin. The potential role of curcumin both in the prevention and therapy of the disease

warrants future studies in a large clinical trial using proper control.

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