Biochemical Pharmacology 83 (2012) 797–804

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Curcumin as anti-endometriotic agent: Implication of MMP-3 and intrinsic apoptotic pathway

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ARTICLE INFO

Article history:
Received 2 November 2011
Accepted 20 December 2011
Available online 29 December 2011

Keywords:
Endometriosis
Matrix metalloproteinase
Curcumin
Apoptosis

ABSTRACT

The disease of reproductive women, endometriosis represents implantation of functional endometrial glands outside uterine cavity. This invasive disorder is associated with dysregulation of matrix metalloproteases (MMP)s and extracellular matrix (ECM) remodeling. In this study, we investigated the role of MMP-3 on apoptosis during endometriosis. We also checked whether curcumin has potency to regress endometriosis by modulating MMP-3 and apoptotic pathway. Mouse model of endometriosis was designed by intraperitoneal inoculation of endometrial tissues to syngeneic female BALB/c. At 15th day, stable endometriotic developments were observed with increased MMP-3 expression. TUNEL positive cells were also found with endometriotic progression, which might resulted from destruction of local immune cells. We speculate that increased MMP-3 activity might be involved in the Fas mediated apoptosis. Curcumin treatment regressed endometriosis by inhibiting NFκB translocation and MMP-3 expression. It also accelerated apoptosis in endometriomas predominantly via cytochrome-c mediated mitochondrial pathway. Involvement of mitochondria in apoptosis was further confirmed by atomic force microscopy (AFM). These results were also supported by our therapeutic study, where curcumin induced apoptosis both by p53 dependent and independent manner, while celecoxib followed only p53 independent pathway. Altogether, our study establishes the novel role of curcumin as a potent anti-endometriotic compound.

1. Introduction

Endometriosis is a gynecological disease representing implantation of functional endometrial glands and stroma outside the uterine cavity. Endometrial epithelial cells produce matrix metalloproteinases (MMPs) during endometrial breakdown while manifestation of the disease is attributed to the attachment and the survival of endometrial cells into peritoneum [1]. An imbalance in extracellular matrix (ECM) homeostasis and apoptosis plays an important role in the etiology of endometriosis [2]. Reactive oxygen species (ROS) mediated inflammation acts as second messenger to activate nuclear factor-κB (NFκB) and transcription of MMPs in endometrial stromal cells [3,4]. MMPs, a family of Zn^{2+} dependent endopeptidases, play a pivotal role in tissue remodeling and invasion in normal physiology [5,6] and in several inflammatory diseases including endometriosis [7,8]. Increased MMP-3 has been reported recently in endometriosis progression [9] though the mechanism is still not clear. MMP-3 can also induce apoptosis possibly by degrading laminin and disruption of cadherins junction formation in mammary epithelial cells [10], however the proper relations between MMP-3 and apoptosis in endometriosis is still unclear.

Apoptosis or "programmed cell death" represents normal cellular mechanism to remove unwanted, damaged cells and is linked with several physiological and pathological processes [11]. Caspase dependent apoptosis follows two pathways, death receptor pathway and mitochondrial pathway. The death receptor pathway comprises of Fas and their respective receptors, which finally activates caspase-8 and -3 [12]. On the other hand, mitochondrial pathway of apoptosis initiates with downregulation of antiapoptotic proteins (Bcl-2, BclxL) and upregulation of pro-apoptotic proteins (Bax, Bad). The decrease in mitochondrial trans-membrane potential is resulted in opening of mitochondrial permeability transition pores to release cytochrome-c (Cyt-c) which eventually activates caspase-9 [13]. Previous reports on endometriosis demonstrated the decreased apoptotic index in patients than normal women [14]. Overexpression of Bcl-2 was found in stromal cells of proliferative eutopic endometriosis compared to normal endometrium [15]. Bax

Abbreviations: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; Cur, curcumin; FasL, fas ligand; Cyt-c, cytochrome-c; MMP, matrix metalloproteinase; ECM, extracellular matrix; NFκB, nuclear factor-κB; JNK, c-jun N-terminal kinase.

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doi:10.1016/j.bcp.2011.12.030

expression was found to be absent in proliferative endometrium resulting in decreased apoptosis during establishment of endometriosis [15]. Additionally, random expressions of Fas were found in eutopic and ectopic endometrial tissues, suggesting less involvement of Fas as an apoptotic regulator [16]. Moreover, increased MMPs in endometriosis have been implicated in the conversion of Fas ligands (FasL) to active soluble forms [17,18]. Expression of FasL in endometrial stromal cells may induce apoptosis in local immune cells, e.g. macrophage, lymphocyte to promote early endometriosis development [19]. Reports have also demonstrated the involvement of p53, a potent inducer of apoptosis, during malignant transformation of endometriosis in human, where p53 staining was found to be negative in benign endometriotic cysts but positive in malignant cysts [20].

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), derived from the rhizomes of *Curcuma* spp, possesses anti-oxidant, anti-inflammatory properties [21]. Higher doses of curcumin were found to be cytotoxic causing inhibition of cell cycle blockage at G2 phase and consequent apoptosis through Cyt-c mediated pathway by increasing mitochondrial permeability [22]. Recently, curcumin has been reported to inhibit activation of NFκB and inhibit c-jun N-terminal kinase (JNK)-mediated pathway [23]. Several reports also confirmed the potency of curcumin to induce p53 mediated direct activation of apoptosis [24]. Moreover, curcumin showed inhibition on ubiquitin-proteasome pathway, which is the principal mechanism in the cell for controlled protein degradation and mitochondrial apoptosis [25]. However there is still no clear report on the effect of curcumin on endometriosis and relevant apoptotic mechanism.

In the present study, we for the first time documented that curcumin is a potent anti-endometriotic agent due to its ability to induce apoptosis. We examined the involvement of MMP-3 and apoptosis during endometriosis development and effect of curcumin thereon. Our finding implicated the role of mitochondrial pathway to induce apoptosis during regression of endometriosis by curcumin treatment. Additionally, comparative analysis of curcumin and celecoxib treatment revealed regulation of apoptosis by p53 dependent and independent pathway to regress endometriosis.

2. Materials and methods

2.1. Chemicals

Casein from bovine milk, Triton X-100, protease inhibitors mixture, Brij 35, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, celecoxib and purified curcumin were obtained from Sigma (Sigma Aldrich Inc, St. Louis, MO, USA). Pre-stained protein molecular weight markers were purchased from Fermentas (Fermentas inc, Washington, DC, USA). Mouse reactive polyclonal anti-MMP-3, anti-Cyt-c, anti-Bcl-2, anti-Bax, anti-Fas, anti-FasL, anti-caspase-3,-9, anti-p65/NFκB, anti-IκB-α, anti-p53, anti-p38, anti-pp38 and anti-β-tubulin antibodies were purchased from Santa Cruz (Santa Cruz Biotechnology inc, California, USA). All other chemicals were purchased from a local company, Sisco Research Laboratories, Mumbai, India.

2.2. Induction of peritoneal endometriosis in BALB/c mice: protective and therapeutic studies

Female adult BALB/c mice of 6–8 weeks old, bred in house with free access to food 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 done modifying Somigliana et al. method using ovariectomized mice. Briefly, on day 0 the donor mice were anesthetized (ketamine 12 mg/kg b.w.) 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 were administered once daily at different doses (e.g. 12, 24, 48 mg/kg body weight) intraperitoneally (i.p.) prior to inoculation of endometrial extract and continued for the next three days to test its protective effects against endometriosis. Mice, 4 each in a group, were sacrificed on day 7 (D7), day 15 (D15) and day 21 (D21) post induction of endometriosis and endometriotic lesions were collected and preserved. All animal experiments were repeated independently for three times ($n = 12$).

For therapeutic study, peritoneal endometriosis was induced without any pretreatment. After 10th day mice were treated with curcumin (48 mg/kg b.w.) or celecoxib (5 mg/kg, twice/day) i.p. for next 5 days. Animals were sacrificed on the 15th day of post endometriosis induction and samples were preserved in −80° for further assays. All animal experiments were repeated independently for three times ($n = 12$).

2.3. Histological studies and TUNEL assay

Endometriotic tissues and uteri obtained from mice were sectioned into 2–3 mm^2 pieces. The tissue samples were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin wax. Approximately, 5 μm thick serial sections were stained with hematoxylin and eosin or subjected to Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) assay by using a commercial reagent kit (DeadEnd™ Fluorometric TUNEL System, Promega, Madision, WI, USA). Fixation, permeabilization, and staining runs were carried out in exact parallel to ensure comparative significance among groups. Images were captured at 100× and 200× in Olympus microscope (1 × 70) using Camedia software (Chicago, MI, USA) (E-20P 5.0 Megapixel) and processed using Adobe Photoshop version 7.0.

2.4. Tissue extraction

Tissues were suspended in PBS containing protease inhibitors, 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.

For preparation of nuclear extract, tissues were minced in ice cold PBS and centrifuged at 1000 × *g* for 5 min. Pellets were resuspended in low salt buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$ and 10 mM KCl) and vigorously mixed after addition of 20 μl of 10% NP-40. Nuclei were collected followed by centrifugation at 12,500 × *g* and resuspending in 50 μl of high salt buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$ and 0.2 mM EDTA and 25% glycerol). Proteins were estimated either by Lowry method or Bradford assay.

To prepare mitochondrial extracts, tissues were washed with PBS and sedimented by centrifugation at 1300 × *g* for 5 min. Cells were then resuspended in a buffer containing 0.3 M sucrose, 1 mM EGTA, 5 mM MOPS, 5 mM KH_2PO_4, 0.1% BSA, pH 7.4, and disrupted using a Dounce homogenizer (three consecutive sets of 20 strokes). The treated cells were centrifuged three times at 2600 × *g* for 5 min. The resulting supernatant was centrifuged at 15,000 × *g* for 10 min to obtain a crude mitochondrial fraction. This mitochondrial fraction was purified by Percoll density

gradient centrifugation and used for immunobloting and atomic force microscopy (AFM).

2.5. Casein zymography

For assay of MMP-3 activities, tissue extracts (50 μg protein/lane) were electrophoresed in 10% SDS-polyacrylamide gel containing 1 mg/ml casein, under non-reducing conditions. The gels were washed twice in 2.5% Triton X-100 and then incubated in stromolysin assay buffer (100 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$, 0.001% (w/v) sodium azide and 0.005% (v/v) Briz 35) for 21 h at 37 °C. Gels were stained with 0.1% Coomassie blue followed by destaining. The zones of caseinolytic activities came as negative staining. Quantification of zymographic bands was done using densitometry linked to proper software (Lab Image, Kapelan Gmbh, Leipzig, Germany).

2.6. 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 20 mM Tris-HCl, pH 7.4 containing 150 mM 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 (1:20,000) for 1.5 h. The bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution.

2.7. Atomic force microscopy

Purified mitochondrial sample (10 μl) were deposited onto freshly cleaved muscovite Ruby Mica sheet (ASTM V1 Grade Ruby Mica from MICAFAB, Chennai, India), followed by drying the sample by using vacuum dryer. AAC mode AFM was performed using a Pico plus 5500 AFM (Agilent Technologies, Tempe AZ, USA) with a piezoscanner maximum range of 9 μm. Micro fabricated silicon cantilevers of 225 μm in length with a nominal spring force constant of 21–98 N/m were used from Nano sensors. Cantilever oscillation frequency was tuned into resonance frequency. The cantilever resonance frequency was fixed at 275.1 kHz. The images (256 × 256 pixels) were captured with a scan size of between 0.5 and 5 μm at the scan speed rate of 0.499 lines/s or 0.474 μm/s and processed using Pico view1.10.1 (9995) software (Agilent Technologies, USA).

2.8. 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; * highly significant $p < 0.001$; ** less significant $p < 0.01$; NS nonsignificant for $p > 0.05$. The statistical analysis of the data was done using GraphPad Instat 3 software. Comparison between groups was done using one-way

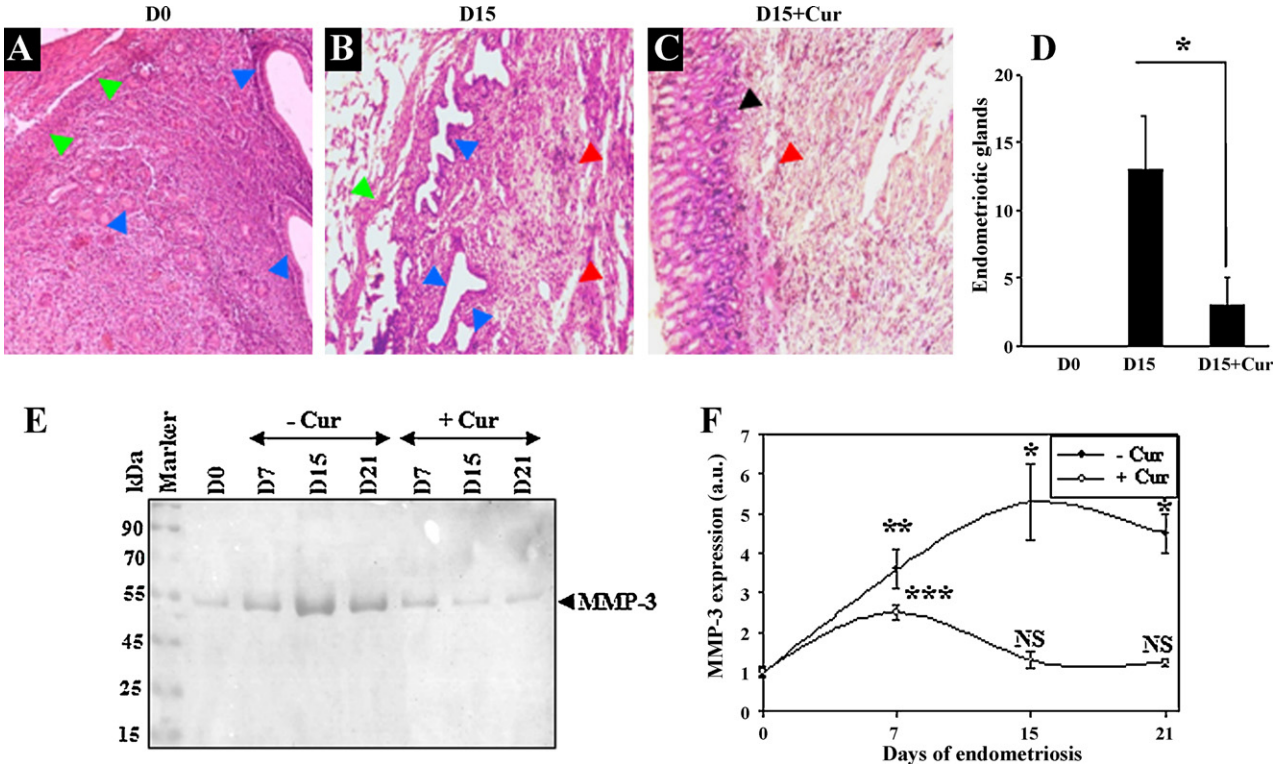

Fig. 1. Pretreatment of curcumin regresses endometriosis by downregulating MMP-3. Curcumin (48 mg/kg b.w.) was administered i.p. for 3 consecutive days during endometrial induction in mice. Endometriosis tissues were collected at different time points from treated and untreated mice. Hematoxylin and eosin staining at 100× magnification of uterine tissues from control mouse (A), endometriosis of day 15 (B), curcumin pretreated day 15 endometriosis (C). Blue arrows represent endometrial glandular epithelium; green arrows represent the stromal region; black arrows for the intestinal mucosal layer and red arrows represent infiltrating inflammatory cells. Graphical representation of mean numbers of peritoneal endometriotic glands, obtained from 4 different samples of each group, during endometriosis and curcumin pretreatment (D). Western blotting as described in Section 2 were performed using equal amounts (100 μg protein) of PBS extracts of different tissues under endometriosis to monitor expression of MMP-3 (E). Graphical representation of arbitrary MMP-3 expression versus days of treatment of above blot and three other blots of independent experiments (F). Values are ± SEM. Sample number $n = 12$. *, $p < 0.001$; **, $p < 0.01$; ***, $p < 0.05$ and NS, nonsignificant versus the appropriate control using ANOVA followed by Student–Newman–Keuls test. Protein band intensities were quantified by densitometric analysis using Lab image software. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

analysis of variance (ANOVA) followed by Student–Newman–Keuls test.

3. Results

3.1. Curcumin downregulates MMP-3 during regression of endometriosis

In order to determine the effect of curcumin on endometriosis, we first confirmed endometriotic development histologically, by hematoxylin and eosin staining. We observed distinct endometriotic glands with stromal and inflammatory cells in peritoneal cavity on 15th day of endometrial induction which suggested functional endometriosis in mouse model (Fig. 1B). Treatment with curcumin showed obliteration of glandular regions although insignificant infiltrating inflammatory cells were observed in intestinal mucosal layer (Fig. 1C). Numbers of peritoneal endometrial glands, which developed after endometroitic induction, were found to be decreased by ~3 fold by curcumin pretreatment (Fig. 1D).

To understand whether peritoneal endometriosis is associated with MMP-3, we performed immunoblotting of MMP-3 using PBS extracts of endometriotic tissues from curcumin treated (48 mg/kg) and untreated mice of different time points. It was observed that expression of MMP-3 gradually increased from 7th day onwards to 15th day of post-endometriosis induction. Endometriotic tissues of day 7 and day 15 displayed ~3 and ~6 fold increase in MMP-3 expressions respectively. Treatment with curcumin shifted the peak of MMP-3 expression from day 15 to day 7. Moreover, decreased expression of MMP-3 was parallel to early regression of endometriosis (Fig. 1E and F).

3.2. Curcumin downregulates MMP-3 by inhibiting NFκB translocation

To ascertain the effect of curcumin's action on MMP-3 expression, we pretreated with curcumin on endometriotic mouse dose dependently. It was evident that curcumin treatment downregulated MMP-3 expression in a dose dependent manner (Fig. 2A and C). Curcumin with the highest dose of 48 mg/kg b.w. successfully reduced MMP-3 expression by ~3 fold as compared to 15th day (D15) endometriosis. Moreover, inhibition of MMP-3 activity was further confirmed by casein zymography, where curcumin pre-treatment regressed MMP-3 activity to control level in a dose dependent manner (Fig. 2B).

To investigate whether increased MMP-3 resulted from increased NFκB translocation into nucleus, immunoblotting was performed against p65 subunit of NFκB using nucleus extract of endometriotic samples (Fig. 2D). Increased translocation of p65 subunit into nucleus with progression of endometriosis indicated increased transcriptional activity of NFκB, which possibly explained elevated expressions of MMP-3. Curcumin treatment decreased MMP-3 expression alongwith reduced NFκB translocation within nucleus. Curcumin treatment also increased IκB-α expression within cytosol that too inhibited nuclear translocation of NFκB (Fig. 2D and E).

3.3. Stimulation of apoptosis by curcumin during regression of endometriosis

Since endometriosis can also be regressed by increased cellular death, we checked *in situ* nuclear degradation in control,

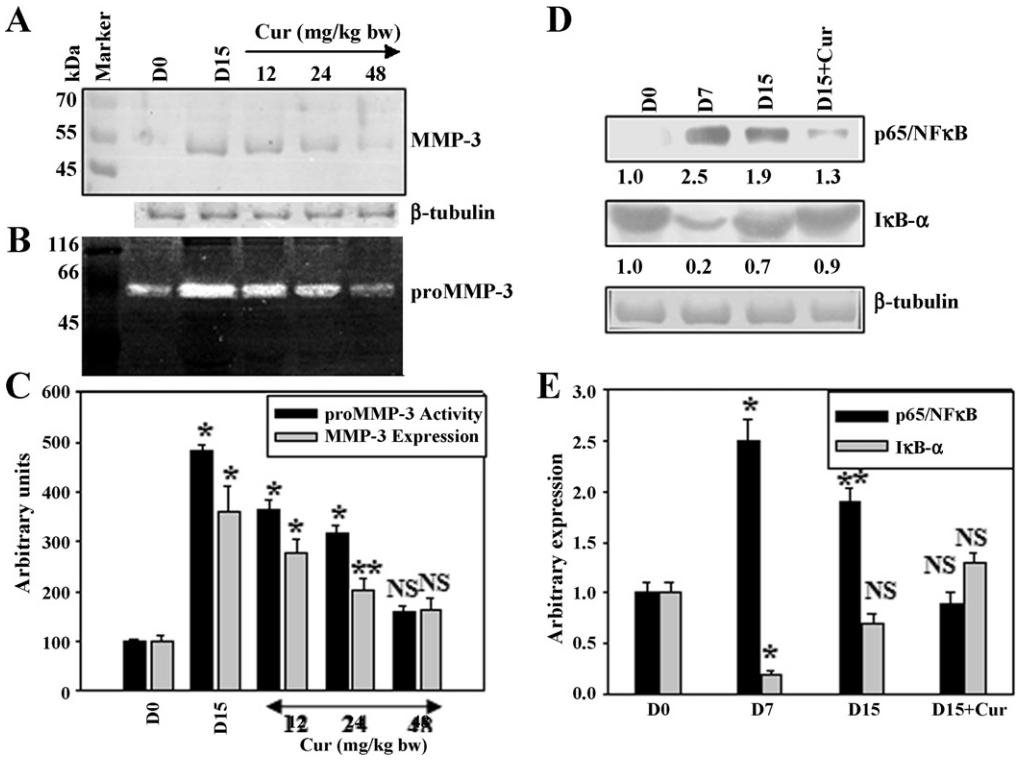

Fig. 2. Curcumin downregulates MMP-3 by inhibiting NFκB translocation during endometriosis regression. To ascertain the effect of curcumin on MMP-3, different doses of curcumin (12, 24, 48 mg/k.g b.w.) were pretreated for 3 days during induction of peritoneal endometriosis. Equal amount of PBS extracts were used to run Western blotting and casein zymography using day 15 endometriotic tissue samples under vehicle and curcumin treatment to monitor the expression (A) and activity (B) of MMP-3. Histographic representation of MMP-3 activity and expressions (C). Expressions of p65 subunit of NFκB (nucleus extract) and IκB-α accumulation within cytoplasm (Tx extract) were analyzed during endometriosis and curcumin treatment by Western blotting using 100 μg of proteins in each lane (D). Histographic representation of band intensities of represented blots of p65/NFκB and IκB-α (E). Values are ± SEM * $p < 0.001$, ** $p < 0.01$ and NS, nonsignificant versus the appropriate control using ANOVA followed by Student–Newman–Keuls test. Protein band intensities were quantified by densitometric analysis using Lab image software from three zymograms and Western blots respectively from independent experiments in each case.

endometriotic and curcumin pretreated endometriotic tissue sections using TUNEL assay. Curcumin treated endometriotic tissues (D15 + Cur) showed ~3 fold of TUNEL positive cells as compared to untreated one (D15) (Fig. 3A(a–c)). Localization of cellular death was restricted only at stroma of 15th day endometriotic tissues whereas curcumin induced cell death found not only in stroma but also glandular regions (Fig. 3B).

To ascertain, whether this cell death has occurred in programmed fashion, we checked the status of the expression of pro-apoptotic and anti-apoptotic mediators. Oscillation of pro- and

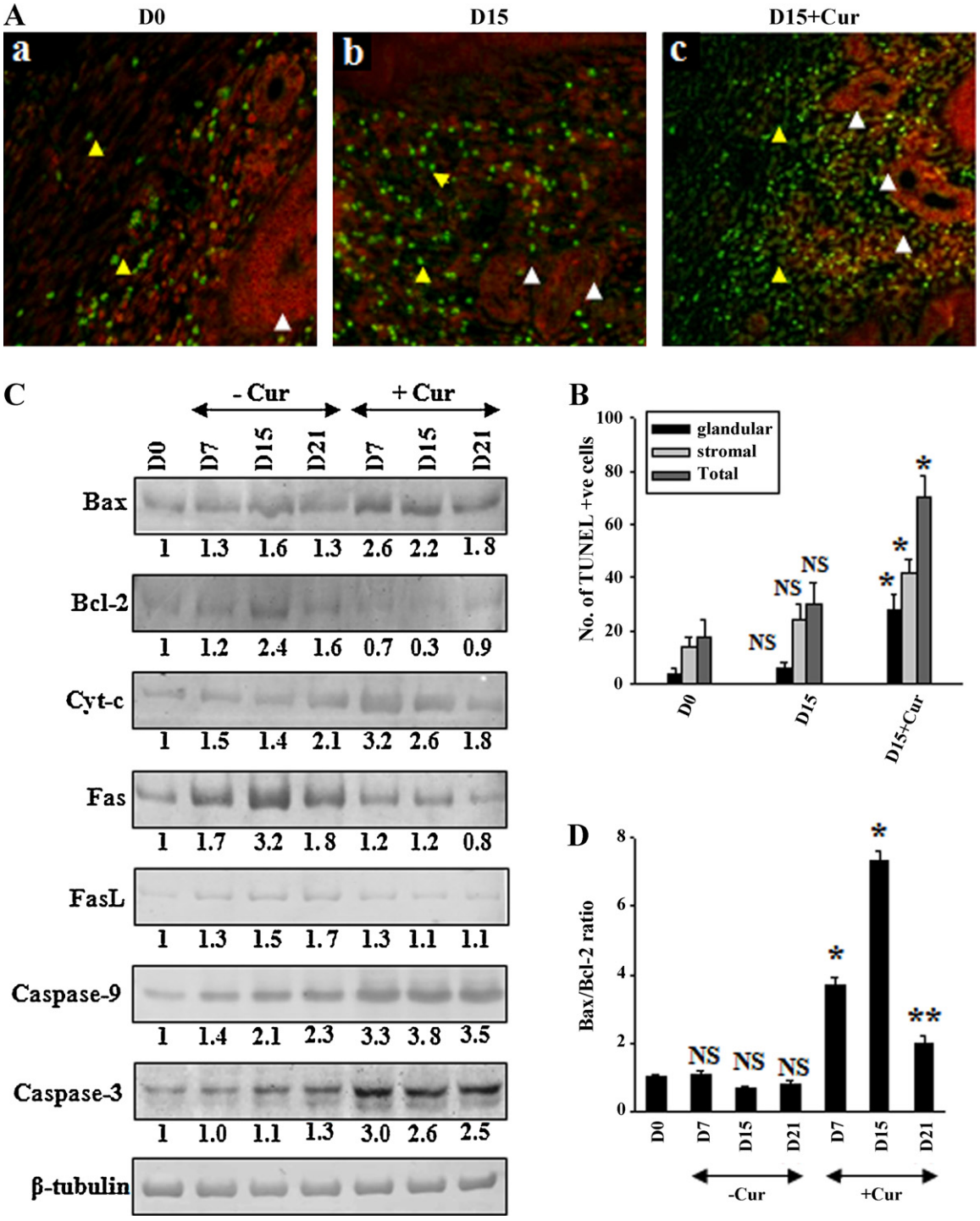

Fig. 3. Curcumin regresses endometriosis by inducing mitochondria mediated apoptosis in mouse model. TUNEL assay was performed to monitor apoptosis in endometriotic tissues of control uteri (Aa), endometriosis at day 15 (Ab), curcumin pre-treated day 15 endometriosis (Ac). DNA fragmentation were observed (green fluorescence) in stromal layer (yellow arrows) and endometrial glands (white arrows) of mouse endometriomas. Micrographs were recorded at 200× original magnification using both FITC and rhodamine fluorescence. Apoptotic nuclei in glandular and stromal regions were calculated from 3 randomly chosen fields per slide in a blinded manner and represented in histogram (B). Western blot analysis of Bax, Bcl-2, Cyt-c, Fas, FasL, Caspase-3, Caspase-9 and β-tubulin using Tx extracts of endometriotic tissues from different groups of mice (C). Histographic representation of Bax/Bcl-2 ratio for apoptotic cells of above Western blots (D). Protein band intensities were quantified by densitometric analysis using Lab image software. Experiments were carried out three times independently and representative Western blots were shown. *, $p < 0.001$; **, $p < 0.01$ and NS, nonsignificant versus the appropriate control using ANOVA followed by Student–Newman–Keuls test.

anti-apoptotic factors during this disease progression indicated the systemic regulation of the cellular death as apoptosis. Endometriotic tissues exhibited increased Bax expression with progression of the diseases, though Bax/Bcl-2 ratio was found to be decreased compared to control (Fig. 3C and D). We also observed increased expressions of Cyt-c and Fas during the development of endometriosis (Fig. 3D).

Western blot analysis revealed that endometriosis occurred both in the presence and absence of curcumin. But curcumin treatment increased Bax/Bcl-2 ratio by ~6 fold compared to control further suggesting the apoptotic role of curcumin (Fig. 3C and D). To understand the mechanism of curcumin's action for induction of apoptosis, we examined both the pathways of apoptosis in curcumin pretreated endometriotic tissues. Curcumin administration induced the expression of Cyt-c and as well as caspase-9, indicated the involvement of mitochondrial pathway. On the other hand, we did not get any induction of Fas or FasL in curcumin treated tissues. Collectively the pathway analysis suggested that curcumin mainly targeted mitochondrial pathway rather than death receptor pathway.

3.4. Curcumin treatment increases mitochondrial size and Cyt-c release

To further investigate the effect of curcumin on mitochondria, we prepared mitochondrial extract from curcumin treated and untreated endometriomas and subjected to atomic force microscopy (Fig. 4A–C). We found increased mitochondrial size during endometriosis; while curcumin treatment increased mitochondrial surface area more significantly, indicating increased permeability and consequent Cyt-c release. To confirm mitochondria mediated apoptosis, Western blotting was performed using purified mitochondrial extracts (Fig. 4E). We observed increased Bax expression within mitochondria during curcumin treatment. Additionally, curcumin treatment caused less Cyt-c expression in mitochondrial extract suggesting Cyt-c release into cytoplasm and elevated apoptosis.

3.5. Differential regulation of apoptosis by curcumin and celecoxib in therapeutic model of endometriosis in mice

To compare the efficacy of curcumin, we used celecoxib in endometriosis in a therapeutic model of endometriosis (Fig. 5A). Morphological observation confirmed healing of endometriosis in presence of curcumin and celecoxib. Distinct lesions, which were observed in the peritoneum on 15th day post-endometriosis, were drastically reduced both in number and volume by curcumin and moderately by celecoxib (data not shown). This finding was also supported by decreased MMP-3 expressions by curcumin and celecoxib (Fig. 5A). We found less expression of p53 protein during endometriotic development while curcumin treatment upregulated p53 expression by ~10 fold while celecoxib treatment showed no upregulation. Additionally, both curcumin and celecoxib were found to activate phosphorylated p38 MAP kinase. Both curcumin and celecoxib triggered the apoptotic responses via increased caspase-9 expressions in comparison to control samples (Fig. 5A).

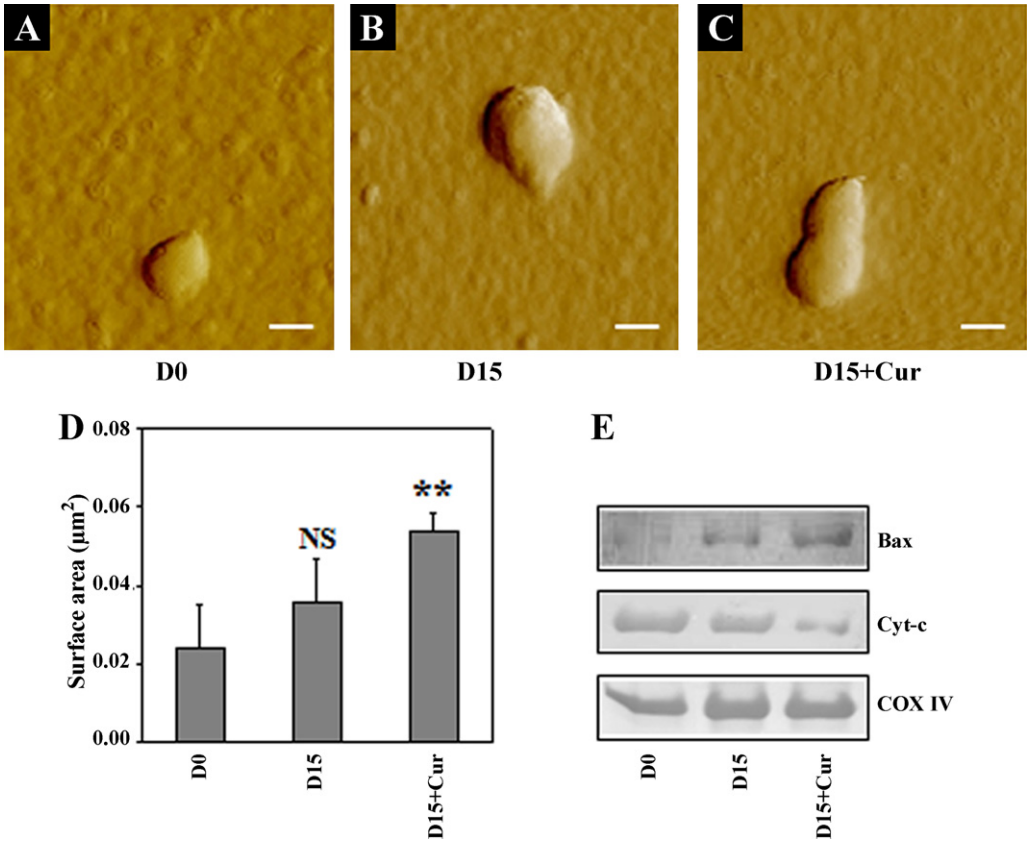

Fig. 4. Curcumin increases Bax translocation, alters mitochondrial size and induces Cyt-c release. Atomic force microscopy was done using purified mitochondrial extract of control uterus (A), day 15 endometriomas (B) and curcumin pretreatment day 15 endometriomas (C). Images were captured in the contact mode after air drying mitochondrial suspension and surface area was analyzed using picoview1.10.1 software. Histographic representation of mitochondrial surface area (D). * $p < 0.001$, ** $p < 0.01$ and NS, nonsignificant versus the appropriate control using ANOVA followed by Student–Newman–Keuls test. Scale bar, 0.1 μm. Immunoblottings for Bax, Cyt-c were performed using purified mitochondrial extract (100 μg/lane). Experiments were carried out three times and representative Western blots were shown (E). COX-IV was used as mitochondrial loading control for protein.

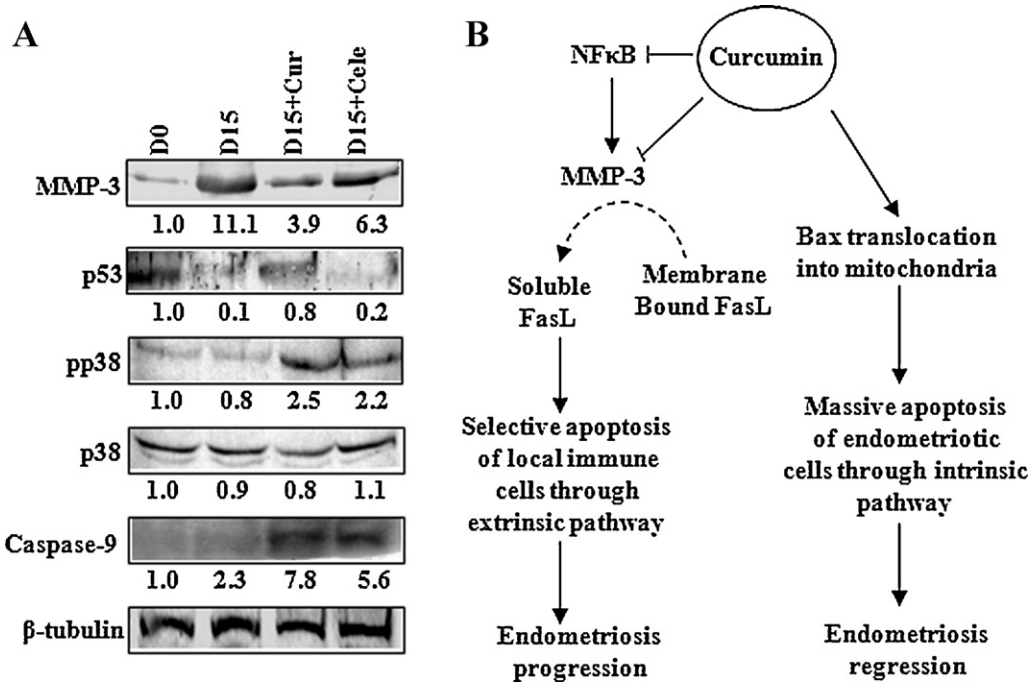

Fig. 5. Comparative regulation of apoptosis during therapeutic treatment by curcumin and celecoxib. Curcumin and celecoxib were tested in therapeutic model of endometriosis in mice as described in Section 2. Endometriotic tissues from different groups of mice were analyzed by Western blotting using mouse reactive anti-MMP-3, anti-p53, anti-phospho-p38, anti-p38, anti-caspase-9 and anti-β-tubulin antibodies (A). Experiments were carried out three times and representative Western blots were shown. Protein band intensities were quantified by densitometric analysis using Lab image software. Schematic diagram of proposed apoptotic regulation of endometriosis with and without curcumin treatment (B). Endometriosis progression is associated with overexpressed MMP-3 via increased NFκB translocation to nucleus. Increased MMP-3 might be involved in FasL solubilization from membrane bound FasL and causes local immune cell death, e.g. lymphocyte, macrophage and neutrophils to progress endometriosis (left panel). In contrast, curcumin regresses endometriosis in endometriotic cells by increasing Bax/Bcl-2 ratio (right panel) and inhibiting MMP-3 expression via NFκB pathway. Hence it causes massive apoptosis of endometriomas and in turn accelerates regression of endometriosis.

4. Discussion

Endometriosis is a common disease of reproductive women that affects10–15% of women population [26]. The role of apoptosis in development of the disease has been considered as one of the major causes. Being an invasive disorder, establishment of endometriosis needs increased MMP activities. MMP supports proliferation and invasion of endometrial exudates within peritoneum; as well MMPs play important roles in cellular functions that are dependent on invasion, migration and differentiation. MMP-9 causes TNF-α mediated proliferation and branching morphogenesis in mammary epithelial cells [6]. In hepatic stellate cells, MMP-2 promotes proliferation and invasion through DDR2 receptor [5]. Therefore, endometriosis could be regressed by such pharmacological agents that can act on MMP expressions as well as apoptosis. Herein, we selected curcumin as it can accomplish both the purposes, as reported by earlier studies [22,27]. In present study, curcumin regressed endometriosis by downregulating MMP-3 alongwith inhibition of NFκB translocation into nucleus. Reports have already established that MMP-3 transcription is modulated by transcription factor NFκB [4]. Therefore, concomitant activation and translocation of NFκB during endometriosis explain MMP-3 overexpression by increased gene transcription. Furthermore, increased IκB-α within cytoplasm during curcumin treatment inhibits translocation of NFκB into nucleus supporting the same hypothesis.

Although endometriosis represents decreased apoptotic index in patients than normal woman [14], on contrary, we found limited increased apoptotic responses during early endometriosis progression. This can be explained by autohealing of the system, as normal menstrual cycle and/or endometriosis are not spontaneous for mouse. Alternatively, apoptosis during early stage of endometriosis represents a systemic process, since early apoptotic responses failed to regress endometriosis adequately. Rather, apoptotic cells are mainly localized at stromal regions, which are the main sites for invasive immune cells. Recently, Lin et al. has reported that macrophage and neutrophil count decreases drastically after early phase of endometriotic induction [19]. Other reports also revealed decreased local immune cells in endometriosis patients compared to normal healthy woman [28]. Therefore, we can hypothesize that induction of apoptosis during early phase of the disease may result from local immune cell death, providing an immune-tolarent environment for endometriotic development. In support, Selam et al. has reported FasL mediated apoptosis of lymphocytes in human endometrial cells [29]. In present study, we also observed increased FasL expression with elevated MMP-3 activities. As MMPs are reported to cleave membrane bound FasL [17], we further propose that increased MMP-3 might be involved to upsurge the bioavailability of soluble FasL inducing apoptosis. Consistent with earlier studies, this report also documents increased Cyt-c release during late phase of endometriosis, suggesting involvement of both the apoptotic pathways. Additionally, decreased Bax/Bcl-2 ratio suggests prognostic significance of apoptosis, indicating resistance to apoptosis and hence progression of the disease.

Although curcumin has long been used as anti-inflammatory agent, recently it is shown to possess anti-carcinogenic activity. Curcumin can induce apoptosis in a JNK dependent manner in colon cancer cell line [23]. It triggers Cyt-c mediated apoptosis by decreasing Bcl-2 and bcl-Xl expressions as reported by Anto et al. [22]. Herein, we report that curcumin treatment regressed endometriosis by drastic induction of apoptosis. More specifically, induction of apoptosis by curcumin follows predominantly Cyt-c mediated mitochondrial pathway. Curcuma has already been reported to induce mitochondrial apoptosis by inhibiting cellular

proteosomal degradation [25]. It can also selectively induce apoptosis in deregulated cyclin D1 expressed cells at G2 phase of cell cycle through involvement of p53 protein [24]. Recently, *Bax* gene has been reported to possess p53 binding site at its promoter region that can directly causes transcriptional activation of the gene [30]. Our study shows similar fact during curcumin treatment, as upregulation of p53 increased Bax expression. Moreover, curcumin triggers mitochondrial apoptosis both by p53 dependent and independent pathways, whereas celecoxib followed solely p53 independent apoptosis. Additionally, She et al. reported p38 kinase mediated activation of p53 and apoptosis through phosphorylation of p53 at serine 15 in mouse epidermal cell line [31]. In support, we revealed the efficacy of celecoxib was moderate compared to curcumin during regression of endometriosis.

In conclusion, our study suggested that activation of MMP-3 play an important role in initial endometriotic development and disease progression. It might selectively induce apoptosis in local immune cells by increasing bioavailability of soluble FasL. Curcumin regresses endometriosis predominantly via mitochondria mediated apoptosis and apoptotic responses include both p53 dependent and independent pathway. We for the first time show that curcumin regresses endometriosis by drastic elevation of mitochondria-mediated apoptotic pathway suggesting therapeutic potential of curcumin as an anti-endometriotic drug. Further studies are warranted to know treatment modalities of endometriosis using proper dose of curcumin.

Acknowledgments

This study was supported by grants MLP13 and IAP 001 of Council of Scientific and Industrial Research, India and GAP209 of Indian Council of Medical Research, New Delhi, India. Authors are grateful to Mr. T. Muruganandan, IICB for assisting AFM and Prof. S. Roy, Director, IICB for constant support and encouragement.

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