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

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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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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