Mitomycin C Induces Apoptosis in Rheumatoid Arthritis Fibroblast-Like Synoviocytes via a Mitochondrial-Mediated Pathway

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Key Words
Mitomycin C • Rheumatoid arthritis • Fibroblast-like synoviocytes • Apoptosis • Mitochondrial pathway

Abstract
Background/Aims: Rheumatoid arthritis (RA) is a systemic chronic inflammatory disease characterised by prominent synoviocyte hyperplasia and a potential imbalance between the growth and death of fibroblast-like synoviocytes (FLS). Mitomycin C (MMC) has previously been demonstrated to inhibit fibroblast proliferation and to induce fibroblast apoptosis. However, the effects of MMC on the proliferation and apoptosis of human RA FLS and the potential mechanisms underlying its effects remain unknown. Methods: Cell viability was determined using the Cell Counting Kit-8 assay. Apoptotic cell death was analysed via Annexin V-FITC/PI double staining and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling. The production of intracellular reactive oxygen species (ROS) was assessed via flow cytometry, and the changes in mitochondrial membrane potential (ΔΨm) were visualised based on JC-1 staining via fluorescence microscopy. The expression of apoptosis-related proteins was determined via Western blot. Results: Treatment with MMC significantly reduced cell viability and induced apoptosis in RA FLS. Furthermore, MMC exposure was found to stimulate the production of ROS and to disrupt the ΔΨm compared to the control treatment. Moreover, MMC increased the release of mitochondrial cytochrome c, the ratio of Bax/Bcl-2, the activation of caspase-9 and caspase-3, and the subsequent cleavage of poly(ADP-ribose) polymerase. Conclusion: Our findings suggest that MMC inhibits cell proliferation and induces apoptosis in RA FLS, and the mechanism underlying this MMC-induced apoptosis may involve a mitochondrial signalling pathway.

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Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory disease characterised by the proliferation and invasion of synovial tissues, leading to progressive joint destruction, deformation and, ultimately, physical disability [1, 2]. Approximately 1% of the global population is affected by RA [3]. Although the precise pathogenesis of RA remains unclear, accumulating evidence suggests that fibroblast-like synoviocytes (FLS) play major roles in both the initiation and the progression of RA [4]. FLS, which are physiologically located in the synovial intimal lining, abnormally proliferate and exhibit aggressive, tumour-like behaviour in patients with RA. These cells produce not only proteases that digest cartilage and bone but also cytokines that mediate inflammatory processes [5, 6]. Moreover, RA FLS exhibit resistance to apoptosis induced by various apoptotic stimuli, further facilitating the hyperplastic growth of FLS and the destruction of articular cartilage [7-9]. Therefore, inhibiting the proliferation of RA FLS and inducing apoptosis of FLS are increasingly under consideration as therapeutic approaches for the treatment of RA.

Mitomycin C (MMC), which is an antitumour antibiotic isolated from the fermented filtrate of *Streptomyces caesporitus*, has widely been used for the treatment of various cancers [10]. Recently, multiple studies have shown that MMC inhibits fibroblast proliferation and induces fibroblast apoptosis [11, 12]. Moreover, our previous studies have demonstrated that topical MMC application may inhibit cell proliferation and induce apoptosis in epidural scar fibroblasts, thereby preventing peridural adhesion after laminectomy [13, 14]. However, whether MMC inhibits human RA FLS proliferation or induces the apoptosis of these cells has yet to be reported.

Apoptosis is a highly regulated process of programmed cell death that is crucial for the maintenance of homeostasis [15]. Apoptosis is initiated by either the death receptor-mediated pathway or the mitochondrial-mediated pathway [16]. A variety of free radicals, such as reactive oxygen species (ROS), are known to cause mitochondrial damage and dysfunction, resulting in the disruption of the mitochondrial membrane potential (ΔΨm) and an increase in the release of cytochrome c from the mitochondria into the cytosol, both of which are regulated by Bcl-2 family proteins. The release of cytochrome c leads to the activation of caspases, thereby inducing apoptosis [17-20].

The aim of the present study was to investigate the effect of MMC on the apoptosis of human RA FLS and the intracellular signalling pathway involved in MMC-induced apoptosis. We examined the effects of different durations of MMC exposure (50 μg/ml) on the following processes: (1) apoptosis of RA FLS, (2) ROS production, and (3) the involvement of the mitochondrial-mediated pathway in MMC-induced apoptosis.

Materials and Methods

**Reagents**

MMC was purchased from Kyowa Hakko Co., Ltd (Tokyo, Japan). Foetal bovine serum (FBS), Dulbecco’s modified Eagle’s medium (DMEM), trypsin-EDTA, penicillin and streptomycin were purchased from Gibco (Grand Island, NY, USA). A Cell-Counting Kit-8 and the Annexin V-FITC Apoptosis Detection Kit were obtained from Dojindo Laboratories (Kumamoto, Japan). A terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL) assay kit, 2’,7’-dichorofluoresceine diacetate (DCFH-DA), and a 5,5’,6,6’-tetrachlоро-1,1’,3,3’-tetraethyl-benzimidazolcarbocyanine iodide (JC-1) detection kit were obtained from Roche (Mannheim, Germany), Beyotime Institute of Biotechnology (Shanghai, China), and BD Pharmingen (San Diego, CA), respectively. Primary antibodies against Bax, Bcl-2, caspase-9, caspase-3, poly(ADP-ribose) polymerase (PARP), COX-IV, β-actin, a horseradish peroxidase-conjugated anti-rabbit secondary antibody and enhanced chemiluminescence (ECL) detection substrate were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibody against cytochrome c was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich.
Isolation and culture of FLS

Synovial tissues from patients with RA were obtained at the time of synovectomy or total knee replacement surgery. All patients fulfilled the American College of Rheumatology criteria for the diagnosis of RA [21]. Informed consent was obtained from each patient, and the procedure was approved by the Ethical Committee of Taishan Medical College. Additionally, all of the studies were performed in compliance with the provisions of the Declaration of Helsinki. Briefly, synovial tissues were minced into small pieces and treated with 2 mg/ml collagenase type I (Sigma) at 37°C for 4 h. After digestion, the tissue suspension was filtered through a 70-μm nylon filter and centrifuged at 400 × g for 10 min. Then the isolated synovial cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidiﬁed atmosphere containing 5% CO₂, and the medium was replaced every three days. FLS from passages three to six were used for all experiments.

Assay of cell viability

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay. Briefly, the cells were seeded in 96-well plates at a final density of 5 × 10³ cells per well in 100 μL of medium. After treatment with increasing concentrations (0, 10, 25, 50, or 100 μg/ml) of MMC for 6, 12, 24, and 48 h, 10 μL of CCK8 solution was added to each well, and the cells were incubated for another 3 h at 37°C. Then, the optical density was measured using an absorbance microplate reader (BioTek, Elx800, USA) at a wavelength of 450 nm. This experiment was performed in triplicate. Cell viability was calculated according to the following formula: optical density of the experimental group/optical density of the control group × 100%.

Determination of apoptosis via flow cytometry

Apoptosis of FLS was measured using Annexin V-FITC/PI double staining and was analysed via flow cytometry. Briefly, the cells were seeded at a density of 1 × 10⁵ cells/well in 6-well plates. After exposure to 50 μg/ml MMC for different durations (6, 12, 24, or 48 h), the cells were harvested with trypsin, washed twice with phosphate-buffered saline (PBS), and resuspended in binding buffer at a concentration of 1 × 10⁶ cells/ml. Then, 100 μL of the cell suspension was incubated in 5 μL of Annexin V-FITC and 5 μL of PI for 15 min at room temperature in the dark. At the end of the incubation, 400 μL of binding buffer was added to the solution. Apoptotic cells were detected using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

TUNEL assay

The TUNEL assay was also performed to assess apoptosis in this study. Apoptotic DNA fragmentation was detected using an In Situ Cell Death Kit according to the manufacturer’s instructions. Briefly, after treatment with MMC, the cells were fixed with 4% paraformaldehyde for 1 h at room temperature. After washing with PBS, the cells were permeabilised with 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. Next, the cells were incubated in the TUNEL reaction mixture for another hour at 37°C in a humidiﬁed chamber shielded from light. Then, the cells were stained with DAPI (Roche, Germany) for 5 min. The incidence of apoptosis was examined under a fluorescence microscope (Nikon, Tokyo, Japan). TUNEL-positive cells were considered to be apoptotic, and DAPI-stained cells were counted to determine the total number of cells in a given field of view.

Measurement of intracellular ROS production

The production of intracellular ROS was detected using the specific fluorogenic probe DCFH-DA. This probe diffuses across the cell membrane and is hydrolysed to DCFH, which is rapidly oxidised to the highly fluorescent compound 2', 7'-dichlorodihydrofluorescein (DCF) in the presence of ROS [22]. The DCF fluorescence intensity is proportional to the ROS levels. Because the generation of ROS is an upstream event that occurs during the process of apoptosis [23], the ROS levels were evaluated at early time points. Briefly, after treatment with 50 μg/ml MMC for 1, 3, 6, 12, or 24 h, the cells were collected and incubated in serum-free DMEM containing 10 μM DCFH-DA for 20 min at 37°C in the dark. Then, the cells were washed three times with serum-free DMEM and resuspended in PBS. The intensity of DCF fluorescence was measured via flow cytometry. The relative intensity of DCF fluorescence was expressed as the percentage of the control group.
Measurement of mitochondrial membrane potential (ΔΨm)

The ΔΨm is an important indicator of mitochondrial function, and disruption of the ΔΨm is suggestive of apoptosis. Loss of ΔΨm was estimated using a Mitochondrial Membrane Potential Detection Kit that included JC-1. In healthy cells with high membrane potentials, JC-1 spontaneously forms aggregates in the mitochondrial matrix and emits red fluorescence. In apoptotic cells with low ΔΨm, JC-1 exists as a monomeric form in the cytoplasm, emitting green fluorescence. The change in fluorescence from red to green occurs when the ΔΨm decreases [24]. Briefly, after 6, 12, 24, or 48 h of exposure to 50 μg/ml MMC, RA FLS were rinsed twice with PBS and incubated at 37°C and 5% CO₂ in a JC-1 working solution for 15 min. The cells were immediately observed under a fluorescence microscope.

Isolation of mitochondrial and cytosolic fractions

The mitochondrial and cytosolic fractions were isolated from RA FLS using a Mitochondria Isolation Kit for Cultured Cells (Thermo Scientific, USA) as described previously [25]. In brief, the cells were incubated in Mitochondria Isolation Reagent A on ice for 2 min and then homogenised using a Dounce homogeniser. Following the addition of Mitochondria Isolation Reagent C, the homogenates were centrifuged at 700 × g for 10 min at 4°C. Then, the supernatant was collected and centrifuged at 12,000 × g for another 15 min at 4°C. The cytosolic and mitochondrial fractions were collected separately for further experiments.

Western blot analysis

Cellular lysates were prepared as described previously [26, 27]. Proteins were resolved using SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Then, the membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature and then incubated over-night at 4°C in antibodies against Bax, Bcl-2, caspase-9, caspase-3, PARP, β-actin or COX-IV (diluted to 1:1000); or an anti-cytochrome c antibody (diluted to 1:500). After three washes with TBST, the membranes were incubated in a horseradish peroxidase-conjugated anti-rabbit secondary antibody at a dilution of 1:2000 for 1 h at room temperature. After washing with TBST, the protein bands were visualised using ECL reagents, and images were acquired using a ChemiDoc Imaging system (ChemiDoc™ XRS + System with Image Lab™ Software; Bio-Rad). The intensities of the protein bands were quantified using Image Lab Software.

Statistical analysis

All of the data were expressed as the means ± standard deviation (SD) of triplicate experiments. Statistical analysis was performed using GraphPad Prism 5.01. The significances of the differences between the means were assessed via one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post-hoc test. P-values of less than 0.05 were considered to be statistically significant.

Results

MMC decreases cell viability in RA FLS

To investigate the effect of MMC on RA FLS, we evaluated cell viability using the CCK8 assay following exposure of the cells to various MMC concentrations (0, 10, 25, 50, or 100 μg/ml) for different durations (6, 12, 24, or 48 h). As shown in Figure 1, MMC significantly reduced the viability of FLS in a dose- and time-dependent manner. Treatment of these cells with 50 μg/ml MMC for 24 h led to a nearly 50% decrease in cell viability. Hence, 50 μg/ml MMC was used in subsequent experiments.

MMC induces apoptosis in RA FLS

To quantitatively examine the effect of MMC on apoptosis in FLS, Annexin V-FITC/PI double staining was measured via flow cytometry. Following treatment with MMC (50 μg/ml for 6, 12, 24, or 48 h), the percentage of apoptotic cells (including early and late apoptotic cells) was found to gradually increase (7.74% ± 1.00%, 16.34% ± 2.86%, 40.50% ± 4.63% and 73.90% ± 4.74%, respectively) compared with the control treatment (5.65% ± 0.77%) (Fig. 2). To further determine the apoptotic effect of MMC on FLS, TUNEL staining was performed. As shown in Figure 3, few TUNEL-positive cells were detected following the
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MMC reduces the viability of human RA FLS. After treatment with increasing concentrations of MMC for 6, 12, 24, and 48 h, the effect of MMC on cell viability was determined using the CCK-8 assay. The data represent the means ± SD of three independent experiments. *p<0.05, **p<0.01, and ***p<0.001 compared with the control group.

MMC-induced intracellular ROS production was detected using the fluorogenic probe DCFH-DA via flow cytometry. After exposure to MMC for the indicated periods, the levels of intracellular ROS were significantly increased within 24 h compared with the control treatment. The maximal increase in the ROS levels was achieved after 3 h of incubation (Fig. 4). These data reveal that MMC induced the production of intracellular ROS, even at 1 h, implying that these cells were subjected to oxidative stress.

MMC induces the production of intracellular ROS

MMC induces the apoptosis of RA FLS. Cells were treated with 50 μg/ml MMC for different durations (6, 12, 24, or 48 h). The cell apoptosis rates were analysed via flow cytometry using the Annexin V-FITC/PI double staining method. (A) Representative flow cytometric plots. Q1 represents necrotic cells; Q2 represents late apoptotic cells; Q3 indicates early apoptotic cells; and Q4 indicates living cells. (B) Graphic representations of the apoptotic rate of each group. The data represent the means ± SD of three independent experiments. *p<0.05 and ***p<0.001 compared with the control group.

control treatment (2.33% ± 1.25%). After treatment with MMC, the percentages of TUNEL-positive cells at 6, 12, 24, and 48 hours were 6.33% ± 2.05%, 21% ± 3.74%, 44.67% ± 6.24%, and 66.67% ± 5.44%, respectively. Taken together, these flow cytometry and TUNEL staining data indicate that MMC significantly induces apoptosis in RA FLS.
MMC induces the loss of \( \Delta \Psi_m \) and the release of mitochondrial cytochrome c

To investigate whether MMC-induced apoptosis is associated with mitochondrial dysfunction, the integrity of \( \Delta \Psi_m \) was assessed using JC-1 fluorescence staining and visualised via fluorescence microscopy. As shown in Figure 5, the red fluorescence gradually decreased and the green fluorescence correspondingly increased following the MMC treatment, indicating the disruption of \( \Delta \Psi_m \) and mitochondrial damage. Loss of the \( \Delta \Psi_m \) initiates the release of cytochrome c into the cytosol, which subsequently activates a caspase cascade that results in apoptosis. Thus, we examined the release of cytochrome c via Western blot analysis. As shown in Figure 6A, MMC treatment caused a time-dependent decrease in...
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the level of cytochrome c in the mitochondria and a corresponding increase in the cytosol, indicating its release from the mitochondria into the cytosol. Taken together, these findings suggest that mitochondrial dysfunction is likely involved in the MMC-induced apoptosis of RA FLS.

**Fig. 5.** MMC induces mitochondrial dysfunction in RA FLS. Cells were exposed to 50 μg/ml MMC for 6, 12, 24, or 48 h, and the mitochondrial membrane potential was monitored by staining with the fluorescent dye JC-1. Red fluorescence represents the mitochondrial aggregated form of JC-1 due to a high ΔΨm, whereas green fluorescence indicates the monomeric form of JC-1 due to a low ΔΨm. Scale bar = 100 μm.

**Fig. 6.** Effect of MMC on the expression of apoptosis-related proteins as determined via Western blot analysis. After exposure to 50 μg/ml MMC for 6, 12, 24, or 48 h, the protein expression levels of Bax, Bcl-2, and cytochrome c were determined via Western blot analysis. The expression levels of β-actin and COX-IV were used as loading controls. The values are presented as the means ± SD of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 compared with the control group.
MMC affects the expression of apoptosis-related proteins in RA FLS

Next, we examined the effect of MMC on the expression of Bcl-2 family proteins, which play an important role in the regulation of mitochondrial permeability. Western blot analysis revealed that MMC treatment increased the level of Bax and decreased the level of Bcl-2 in a time-dependent manner. Therefore, the ratio of Bax to Bcl-2 was increased (Fig. 6B). The caspase family is believed to play a central role in mediating various apoptotic responses. To confirm the induction of the apoptotic pathway, we examined the activation of caspase-9, caspase-3, and PARP. As shown in Figure 7, MMC induced a significant increase in cleaved fragments of caspase-9 and caspase-3. PARP, which is a substrate of caspase-3, was also cleaved in response to MMC. These findings suggest that MMC induced apoptosis of RA FLS via the mitochondrial-mediated apoptotic pathway.

Discussion

MMC is an alkylating agent that displays potent anti-tumour activity. MMC impairs DNA replication and prevents protein synthesis, suppressing the proliferation of fibroblasts and epithelial cells and leading to the decreased development of granulation tissue and reduced scar formation, thereby diminishing the negative impacts of fibroblast proliferation [28-30]. Due to the recent increase in relevant studies and clinical trials, MMC has been successfully applied to decrease scar formation during postoperative wound healing in the fields of ophthalmology and spinal surgery [14, 31, 32]. Because FLS are homologous to fibroblasts,
the primary objective of this experimental study was to investigate the effect of MMC on the apoptosis of FLS derived from RA patients and the mechanism underlying MMC-induced apoptosis.

Previous studies have confirmed the effectiveness of MMC in promoting antiproliferation and apoptotic signalling in fibroblasts [11]. In this study, we detected an influence of MMC on human RA FLS. The CCK-8 assay results showed that MMC effectively inhibited the proliferation of FLS in a dose- and time-dependent manner compared with the control treatment. In addition, MMC exposure caused an increase in the percentage of Annexin V-positive and TUNEL-positive cells. These results demonstrate that MMC inhibited the proliferation of human RA FLS and induced the apoptosis of these cells. In previous in vitro studies, fibroblasts are usually exposed to MMC at a high dose for short durations [13, 33]. However, a relatively low concentration of MMC treatment for longer periods can also be found [34, 35]. Besides, RA FLS act like tumour cells and exhibit resistance to apoptosis compared with normal fibroblasts [5-7]. Based on these and the CCK-8 assay results, RA FLS were treated with MMC (50 μg/ml) for long periods in the present study.

Apoptosis is an ordered and orchestrated cellular suicide process that occurs under physiological and pathological conditions [36]. Oxidative stress is considered to be important for the promotion of apoptosis in response to a variety of apoptotic stimuli. ROS play essential roles in the oxidative stress response. Cells constitutively generate these molecules, which play important roles in redox balance and physiologic cell function. However, excessive ROS production can directly lead to mitochondrial damage and dysfunction, followed by a series of mitochondria-associated events, including apoptosis [17, 37, 38]. In our previous study, we found that MMC induces the apoptosis of epidural scar fibroblasts by elevating the production of intracellular ROS [13]. Though using different tissue types, common mechanisms may be shared. In agreement with these previous results, our current findings showed that MMC significantly increased the production of intracellular ROS in RA FLS, and the ROS levels peaked after 3 h of incubation. Therefore, excessive ROS generation is likely involved in MMC-induced apoptotic cell death in RA FLS.

It is well known that apoptosis occurs via two primary pathways: the extrinsic pathway, which is associated with cell death receptors and their ligands on the cellular surface, and the intrinsic pathway, which is dependent on mitochondria. The latter has been considered to be the predominant apoptosis-inducing pathway [16, 39]. Previous studies have demonstrated that mitochondrial dysfunction causes the collapse of ΔΨm, which results in mitochondrial permeability transition pore (mPTP) opening, enabling the release of cytochrome c from the mitochondria into the cytosol [40, 41]. The present study demonstrated that MMC exposure caused a loss of ΔΨm in FLS and obvious clear translocation of cytochrome c from the mitochondria to the cytosol after MMC treatment. The level of cytochrome c decreased in the mitochondria and correspondingly increased in the cytosol. These data indicate that MMC caused mitochondrial damage to RA FLS.

The mitochondria-dependent apoptotic pathway is regulated by the Bcl-2 family of proteins, including Bax and Bcl-2 [25, 42]. Bax, which is a pro-apoptotic member of the Bcl-2 family, promotes the opening of the mPTP, causing the release of cytochrome c, whereas Bcl-2, which is an anti-apoptotic member of the Bcl-2 family, inhibits the formation of the mPTP, blocking cytochrome c release [43]. An increase in the Bax/Bcl-2 ratio has been demonstrated to promote apoptosis by directly activating the mitochondrial apoptotic pathway [44]. As demonstrated in the present study, the expression of Bcl-2 decreased, while that of Bax increased, resulting in an elevation in the Bax/Bcl-2 ratio, suggesting that MMC promoted the apoptosis of RA FLS via the mitochondrial-dependent apoptotic pathway.

Once released into the cytosol, cytochrome c triggers the activation of caspase-9. Subsequently, this initiator caspase activates downstream caspase-3, which is a critical executioner of apoptosis. Following caspase activation, an increasing number of cellular substrates, including the DNA repair protein PARP, are degraded or cleaved, resulting in cell death [19, 20, 45]. Multiple studies have shown that MMC causes to a notable induction of apoptosis via the mitochondrial-dependent apoptotic pathway [46, 47]. Our findings are
consistent with these results, which showed that MMC significantly induced the activation of caspases-9 and caspase-3. Further investigation revealed a time-dependent increase in PARP cleavage in the MMC-treated cells. Our data suggest that the caspase-dependent mitochondrial signalling pathway is involved in the mechanism underlying the MMC-induced apoptosis of RA FLS.

In conclusion, our study is the first report of the effective inhibition of FLS proliferation using MMC in a dose- and time-dependent manner, causing apoptotic cell death via excessive ROS generation and the mitochondrial-dependent pathway. These findings suggest that MMC may exert beneficial effects by postponing the process of joint destruction and relieving the pain experienced by RA patients.

Disclosure Statement

The authors declare no conflicts of interest.

Acknowledgments

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