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Mcl-1 Is Essential for the Survival of Synovial Fibroblasts in Rheumatoid Arthritis

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Mcl-1 is a Bcl-2-family, antiapoptotic molecule that is critical for the survival of T and B lymphocytes and macrophages; however, its role in nonhemopoietic cells remains to be fully elucidated. The current study focuses on the role of Mcl-1 in rheumatoid arthritis (RA). Mcl-1 was strongly expressed in the synovial lining and was increased in the sublining fibroblasts of patients with RA, compared with control synovial tissue. The expression of Mcl-1 in sublining fibroblasts correlated with the degree of inflammation and TNF-α, and IL-1β treatment of cultured synovial fibroblasts resulted in the increased expression of Mcl-1 at the mRNA and protein levels. Mcl-1 was critical for the survival of RA synovial fibroblasts, because the forced reduction of Mcl-1 using a Mcl-1 antisense-expressing adenoviral vector induced apoptotic cell death, which was mediated through Bax, Bak, and Bim. These observations document a critical role for Mcl-1 in protecting against apoptosis in RA and suggest that Mcl-1 is a potential therapeutic target in this disease. The Journal of Immunology, 2005, 175: 8337–8345.

Rheumatoid arthritis (RA) is characterized by the infiltration of lymphocytes and macrophages in the synovium, hyperplasia of synovial fibroblasts, enhanced angiogenesis, and destruction of cartilage and bone (1). The mechanisms that contribute to the persistence of chronic inflammation observed in RA are poorly characterized; however, proliferation of synovial fibroblasts and their resistance to apoptosis may be crucial in the pathogenesis of this disease (2). Histological evidence of apoptosis has not been observed in RA synovial tissue examined by electron microscopy (3, 4). Joint inflammation and destruction in a variety of animal models, including streptococcal cell wall-induced arthritis, collagen-induced arthritis, the human T cell leukemia virus 1 tax transgenic model, and RA explants in SCID mice, have each been ameliorated by enhanced apoptosis (5–7), suggesting that increased apoptosis may be associated with an improved clinical outcome. Therefore, a better understanding of the mechanisms that regulate apoptosis in RA, particularly in synovial fibroblasts, is important, not only to better understand the pathogenesis but also to develop more effective therapeutics.

Mcl-1 was first cloned from the ML-1 (the human myeloid leukemia cell line) as an early-induction gene following treatment with 12-O-tetradecanoyl-phorbol-13-acetate (8, 9). Structurally and functionally, Mcl-1 belongs to the prosurvival Bcl-2 subfamily that also includes Bcl-xL, Bcl-2, and A1 (10). Mcl-1 protein expression is widespread in vivo in normal human tissues. Tissues with high expression of Mcl-1 often have less or no Bcl-2 expressed, suggesting a unique function(s) for Mcl-1, compared with Bcl-2 (11). Increased expression of Mcl-1 is observed during the differentiation of myelomonocytic cells and may be important for survival during this process (12–14). Mice transgenic for Mcl-1 demonstrated the enhanced viability of a wide range of hemopoietic cell types, including B and T lymphocytes, CD11b-positive myeloid cells, including monocytes, macrophages, and polymorphonuclear leukocytes, at both immature and mature stages of differentiation (15). The essential in vivo roles of Mcl-1 have been difficult to define because Mcl-1 deficiency results in peri-implantation embryonic lethality (16). However, Mcl-1 may be essential for the survival of hemopoietic cells, because apoptosis is rapidly triggered by antisense (AS) depletion of Mcl-1 in primary human macrophages, differentiating U937 cells, and in human polymorphonuclear cells (17–19). Recent studies also demonstrated that Mcl-1 is essential to the development and maintenance of B and T lymphocytes and the survival of hemopoietic stem cells (20, 21). Our published data demonstrated that both the PI3K/Akt-1 pathway and the STAT3 pathway controlled the expression of Mcl-1 in primary human macrophages and that Mcl-1 was essential for macrophage survival (17, 22).

In addition to the important role of Mcl-1 in the survival of hemopoietic cell types, accumulating data suggest that Mcl-1 may be necessary for the survival of nonhemopoietic cells (23–25). This study characterizes the expression of Mcl-1 in the RA joint. Mcl-1 is highly expressed in synovial fibroblasts, and the reduction of Mcl-1 promotes apoptotic cell death. The apoptotic cell death induced by the reduction of Mcl-1 in RA synovial fibroblasts was mediated by Bim, Bax, and Bak. These observations suggest that Mcl-1 may be an important therapeutic target in chronic inflammation, such as RA.

Materials and Methods

Materials

LY 294002 and polymyxin B sulfate were obtained from Sigma-Aldrich. Human TNF-α and IL-1β were from R&D Systems. Propidium iodide (PI) was from Roche Molecular Biochemicals, and rhodamine 123 (Rh123) was...
from Molecular Probes. Caspase 9 and caspase 3 fluorescent assay reagents were from Biovision.

Patients and cell and tissue preparation

Synovial tissue was obtained at the time of arthroplasty on patients diagnosed with RA (n = 28) or osteoarthritis (OA) (n = 18). The diagnosis was based on American College of Rheumatology classification criteria for RA and OA, respectively (26, 27). Synovial tissue was also obtained from the joints of arthritis-free controls (AFCs) (n = 9) at the time of autopsy from the National Disease Research Interchange (Philadelphia, PA). The Institutional Review Board at Northwestern University approved all experiments on human cells and tissues.

Immunohistochemical analysis

Five-micrometer sections of synovial tissues fixed in 10% neutral buffered formalin were deparaffinized and blocked in 10% goat serum. The sections were incubated with either rabbit anti-Mcl-1 Ab or normal rabbit IgG (Sigma-Aldrich). A biotinylated goat anti-rabbit secondary Ab (BioGenex), followed by alkaline phosphatase (BioGenex) conjugated to streptavidin, was used to detect primary Ab complexes. Visualization was accomplished using the fast red-alkaline phosphatase substrate kit (BioGenex) and counterstaining with hematoxylin. The synovial lining thickness (median cell number), inflammation score (1–4 scale; estimated by the degree of sublining inflammatory cell infiltrate composed of macrophages, lymphocytes, and neutrophils), and the percentage of Mcl-1-positive cells was scored by a pathologist who was blinded to the elements of the study as described previously (28–30). Briefly, the inflammatory score was defined as follows: 1, a few scattered inflammatory cells; 2, clusters of inflammatory cells; 3, diffuse infiltrate of inflammatory cells; and 4, dense sheets of inflammatory cells, including lymphoid follicles. Staining intensity, which was scored on a 0–4 scale, can be altered by many factors. Consequently, steps were taken to minimize the effect of known variables. These steps included staining for each Ab in batches to ensure consistency in Ab concentrations, application time, temperature, and humidity. Relative staining intensity was semiquantitatively assessed as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining; and 4+, intense staining. The range of staining intensity was initially assessed, and cases with representative staining intensities were used as standards to compare and categorize the remaining cases.

Culture and infection of RA synovial fibroblasts

RA and OA synovial fibroblasts were isolated and used between passages 3 and 9 as described previously (29, 31). The RA synovial fibroblasts were infected with adenoviral (Ad) vectors expressing β-galactosidase (Adβgal), the Mcl-1 AS (AdMcl-1 AS), DiRed-Mcl-1 (AdMcl-1), or Bcl-xL (AdBcl-xL). RA synovial fibroblasts were infected at the indicated multiplicity of infection (m.o.i.) using DMEM with 0.5% FCS for 6 h (for AdMcl-1 AS and its control) or 24 h (all other vectors and their controls). The cells were then washed with PBS, DMEM containing 10% FBS was added, and the cells were cultured for 24–72 h as indicated in the figures.

Intracellular staining of Mcl-1 of synovial fibroblasts

Cultured synovial fibroblasts were blocked for 1 h at room temperature in 50% human serum (32). After blocking, synovial fibroblasts were fixed in 4% neutral buffered formalin, permeabilized with 0.1% Nonidet P-40 (Sigma-Aldrich), blocked overnight at 4°C in 90% goat serum, and incubated at 4°C for 3–4 h with either rabbit anti-Mcl-1 Ab (Santa Cruz) or control rabbit IgG (Sigma-Aldrich). Cells were then incubated with FITC-labeled goat anti-rabbit Ab (Jackson ImmunoResearch Laboratories) at 4°C for 1–2 h. Mcl-1 expression was determined in the synovial fibroblasts by flow cytometry, and intracellular Mcl-1 was quantified by the mean fluorescence intensity.

Construction of Ad vectors

To construct Ad vector expressing Mcl-1 AS, Mcl-1 cDNA was released from pCI-Mcl-1 (provided by Dr. S. Nimer, Memorial Sloan-Kettering Cancer Center, New York, NY) by EcoRI, then ligated to a pCR2.1 (In-vitrogen Life Technologies) vector in the EcoRI site. After screening by enzyme digestion, the vector expressing the full-length Mcl-1 AS was confirmed by DNA sequencing. The Mcl-1 AS was cloned between the HindIII and BglII sites of pCMV-Genvec (33) to generate pGenvec Mcl-1 AS. The Mcl-1 AS was cut out of pCMV-Genvec containing terminal repeated CMV promoter, Mcl-1 AS, and SV40 polyadenylation sequence (in that order) were excised and ligated to the 4–1000 bp unit fragment of the Ad DNA Ads5-309/356. The ligated DNA was subsequently transfected into subconfluent 293 cells to generate the Ad vector expressing Mcl-1 AS (34).

To construct an Ad vector expressing DiRed-Mcl-1 fusion protein, 5′ primer with HindIII cutting site and 3′ primer with BamHI cutting site were designed, the PCR product using pC1-Mcl-1 as a template was cut with HindIII and BamHI and the fragment was cloned into the HindIII and BamHI sites of pDsRed2-C1 vector (BD Biosciences) to generate pDsRed-Mcl-1. The NheI-BamHI fragment from pDsRed-Mcl-1 containing the fusion vector was cloned into pDONR-CMV by homologous recombination. The NheI/BamHI sites of pDONR-CMV were then used to get pDONR-CMV-DsRed-Mcl-1. This vector was used to generate the Ad vector expressing DiRed-Mcl-1 (AdMcl-1) using the BD Adeno-X Expression System 2 (BD Biosciences) according to the manufacturer’s instructions.

Apoptosis assessment

The percentage of apoptotic cells was determined by analysis of subdiploid DNA (<2N), analyzed by flow cytometry as described previously (17), or by annexin V-PE staining, which was quantified by flow cytometry as described previously (35). Caspase 9 or caspase 3 activity was assessed using fluorogenic substrates as described previously (17). Mitochondrial dysfunction was determined using the cationic lipophilic green fluorochrome Rh123 as described previously (36). The disruption of mitochondrial transmembrane potential (ΔΨm) is associated with decrease of Rh123 retention measured by a reduction of fluorescence.

Western blot analysis

Whole-cell extracts were prepared from RA synovial fibroblasts that were treated as indicated in Results. Extracts were electrophoresed on SDS-PAGE 12.5% polyacrylamide gels and transferred to Immobilon-P (Millipore) by semidyblotting. The membranes were then incubated overnight at 4°C in PBS/Tween/milk with the indicated Abs: anti-Mcl-1 (Santa Cruz), anti-caspase 9 (Calbiochem) and anti-caspase 3 (BD Transduction Laboratories), anti-cytochrome c (BD Pharmingen), mouse anti-Bax (Sigma-Aldrich), rabbit anti-Bak (Upstate Biotechnology), mouse anti-cytochrome oxidase subunit IV (Molecular Probes), or anti-tubulin (Sigma-Aldrich). Membranes were washed in PBS/Tween/milk and incubated with either donkey, anti-rabbit, or anti-mouse secondary Abs conjugated to HRP (Amersham Biosciences). Visualization of the protein bands was performed using the Enhanced Chemiluminescence Plus kit (Amersham Biosciences) according to the manufacturer’s instructions.

Real-time PCR

Total cellular RNA was extracted and used for reverse transcription. Real-time PCR was performed using primers and probes for Mcl-1 and GAPDH, TaqMan Master Mix, and the TaqMan Gene Expression Assays system using a 7500 Real-Time PCR System, all from Applied Biosystems. The relative expression of Mcl-1 was determined using the ΔΔCt method.

Confocal microscopy

RA synovial fibroblasts were plated on glass slides and infected with either Ad Mcl-1 AS or control virus at 100 moi, cultured for 24 h, and then incubated with Mitotracker Red (50 nM; CMXRos; Molecular Probes) for 30 min. The AdMcl-1 AS-infected floating cells were harvested onto slides using a cytospin centrifuge. Subsequently, the slides were stained with anti-Bax as described previously (37, 38). Briefly, RA synovial fibroblasts were fixed in 2% formaldehyde in PBS, permeabilized with 0.1% Triton X-100, and incubated with mouse anti-Bax followed by FITC-labeled anti-mouse H and L chain (H+L). Confocal microscopy was performed with an LSM510 laser-scanning confocal microscope (Zeiss).

Transfection of short-interfering RNA (siRNA) into RA synovial fibroblasts

The siRNA to Bax (Bax siGenome SMARTPool), Bak (Bak1 siGENOME SMARTPool), and Bim or nonspecific control siRNA were from Dharmacon. Lipofectamine and 5 μl of Lipofectamine 2000 (Invitrogen Life Technologies) was used for the transfection according to the manufacturer’s instructions. In brief, for each well of the six-well culture plate, 5 μl of Lipofectamine and 5 μl of siRNA in 1 ml of serum free-Opti-MEM (final concentration, 100 μM) were used. Four to 6 h after the transfection, 1 ml of 1% FBS in DMEM was added (final 0.5% serum), and cells were further cultured for 72 h. Transfected cells were then infected with AdMcl-1 AS at 200 moi, and the cells were cultured for an additional 24 h. The cells were harvested, Western blot analysis was used to detect the protein levels, and apoptosis was determined by annexin V staining.
**Statistical analysis**

The results are expressed as the mean ± 1 SEM. Differences between groups were analyzed using a Student two-tailed t test. Correlations were determined by regression analysis.

**Results**

The expression of Mcl-1 in synovial joint tissues correlates with inflammation in RA, OA, and normal synovial tissues

Mcl-1 was highly expressed in the synovial lining, fibroblasts in the sublining, and in blood vessels of patients with RA (Fig. 1). Mcl-1 was also detected in the tissues from patients with OA and in the tissues from AFCs (Fig. 1). The percentage of Mcl-1-positive fibroblast-apparing cells in the sublining, identified by phenotype as described previously (39), was significantly greater in RA, compared with OA and normal, synovial tissues (Table I). Because the inflammatory scores were different between RA and the OA or AFCs, the relationship of inflammation with the expression of Mcl-1 was examined. The intensity of Mcl-1 staining in the lining and the percentage of Mcl-1 positive fibroblasts in sublining, correlated (p < 0.05 and p <0.001, respectively) with the inflammation when RA, OA, and AFC synovial tissues were examined (Table I), suggesting that the proinflammatory cytokines might contribute to the expression of Mcl-1 in the synovial tissues.

Expression of Mcl-1 is higher in RA, compared with OA synovial fibroblasts

The results of immunohistochemistry suggested that Mcl-1 was increased in RA synovial fibroblasts. Immunoblot analysis demonstrated Mcl-1 in the synovial fibroblasts from patients with both RA and OA (Fig. 2A). Mcl-1 expression was greater (p < 0.05) in RA synovial fibroblasts (n = 4), compared with those obtained from patients with OA (n = 3), when the bands identified by immunoblot were measured by densitometry and normalized for the expression of tubulin (Fig. 2, A and B). Further study, using fibroblasts from additional patients (n = 7), quantified intracellular Mcl-1 staining by flow cytometry. The RA synovial fibroblasts expressed more Mcl-1 (p < 0.05), compared with synovial fibroblasts from patients with OA (Fig. 2C) or from AFCs (data not shown). There was no significant difference in the expression of Mcl-1 determined by intracellular staining between AFC and OA synovial fibroblasts (data not shown). These observations suggest that Mcl-1 expressed in synovial fibroblasts might be relevant to the pathogenesis of RA.

Because the expression of Mcl-1 in sublining synovial fibroblasts correlated with the inflammatory scores, the effects of proinflammatory cytokines were examined. Incubation of RA synovial fibroblasts with TNF-α or IL-1β increased the expression of Mcl-1 in the RA synovial fibroblasts at the protein level (Fig. 2D). The effect of TNFα and IL-1β on Mcl-1 mRNA expression in RA and OA synovial fibroblasts was examined. Both TNFα and IL-1β resulted in a modest but significant (p < 0.05) increase of Mcl-1 in RA synovial fibroblasts (Fig. 2E). The response to each cytokine was greater (p < 0.02–0.01) in OA synovial fibroblasts, compared with RA synovial fibroblasts (Fig. 2E). These observations suggest that the proinflammatory environment in the RA joint might contribute to the increased expression of Mcl-1 in synovial fibroblasts.

Mcl-1 expression is essential to the survival of RA synovial fibroblasts

To determine the function of Mcl-1 directly in the RA synovial fibroblasts, an Ad vector was constructed expressing full-length human Mcl-1 AS. Infection of RA synovial fibroblasts with the vector expressing the Mcl-1 AS dramatically decreased the expression of Mcl-1 determined by immunoblot analysis (Fig. 3A). Decreased Mcl-1 expression using the Mcl-1 AS resulted in the loss of mitochondrial transmembrane potential (ΔΨm) (Fig. 3B) and cell death (C), as determined by the inability to exclude PI. The mode of cell death was apoptosis (Fig. 3D), defined by analysis of sub-diploid DNA. Confirming the importance of Mcl-1, infection of RA synovial fibroblasts with an Ad vector expressing Mcl-1 protected the cells from apoptosis (Fig. 3E). Additionally, infection with a vector expressing Bcl-xL also protected the RA synovial fibroblasts from apoptosis (Fig. 3E). These observations suggest that Mcl-1 is essential for the survival of RA synovial fibroblasts, and that Bcl-xL may substitute for Mcl-1.

Down-regulation of Mcl-1 results in the apoptosis of RA synovial fibroblasts through disruption of mitochondrial function

To further understand the potential mechanisms contributing to the apoptosis induced by down-regulation of Mcl-1 in RA synovial fibroblasts, the cytosolic and heavy membrane fraction enriched in mitochondria were isolated 24 h after Ad vector infection of RA synovial fibroblasts. Following infection with the Ad vector expressing the Mcl-1 AS, cytochrome c was decreased in the heavy membrane fraction containing the mitochondria and was released into the cytosolic fraction (Fig. 4A). Additionally, infection with the AdMcl-1 AS resulted in activation of caspase 9 and caspase 3, determined by cleavage of procaspase 9 and procaspase 3 by immunoblot assay (Fig. 4B), and up-regulation of caspase 9 and caspase 3 activity, using specific fluorescent substrates (C and D). These observations suggest that the reduction of Mcl-1 in RA synovial fibroblasts results in the release of cytochrome c and the subsequent activation of caspase 9 and caspase 3, which results in apoptosis.

Bax mobilizes to the mitochondria following the reduction of Mcl-1

Further studies were conducted to characterize the potential role of Bax in the apoptosis of RA synovial fibroblasts induced by down-regulation of Mcl-1. Following the reduction of Mcl-1 using the AdMcl-1 AS, Bax was reduced in the cytosolic fraction and increased in the heavy membrane fraction enriched in mitochondria.

**FIGURE 1.** The expression of Mcl-1 in RA and AFC synovial tissues. Synovial tissues from 11 RA, 9 OA, and 8 AFC controls were stained with Abs to Mcl-1 (red) or normal rabbit IgG (red) and counterstained with hematoxylin (blue). Presented are representative tissues from a patient with RA, OA, and an AFC. SL, Synovial lining; V, blood vessel. Magnification, ×200.
The translocation of Bax to mitochondria was further confirmed by confocal microscopy (Fig. 5B). In the RA synovial fibroblasts infected with control Adβgal, the Bax and the mitochondria were distributed in distinctive patterns within the cell. Although the mitochondria were apparent throughout the cell, Bax was distributed diffusely with enrichment around the nucleus. This pattern was observed with two different anti-Bax Abs. After the depletion of Mcl-1 by Mcl-1 AS, Bax and the mitochondria shared a similar pattern within the cell, with colocalization determined by the yellow color in the combined images. These observations demonstrate that, in RA synovial fibroblasts, following the reduction of Mcl-1, Bax translocates from the cytosol to the mitochondria.

Bax, Bak, and Bim are involved in apoptosis of RA synovial fibroblasts

Because Bax and Bak are critical proapoptotic molecules, to document their potential involvement in Mcl-1 depletion-induced apoptosis in RA synovial fibroblasts, siRNA to Bax and Bak were used to suppress their expression in RA synovial fibroblasts. The siRNA to Bax or Bak dramatically suppressed the expression of Mcl-1.

<table>
<thead>
<tr>
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<th>RA (n = 11)</th>
<th>OA (n = 9)</th>
<th>AFC (n = 8)</th>
<th>t Test Between RA and OA</th>
<th>t Test Between RA and AFC</th>
<th>Correlation with Inflammation (RA + OA + AFC) (n = 28)</th>
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<tr>
<td>Inflammation score (1–4)</td>
<td>2.43 ± 0.12</td>
<td>1.44 ± 0.14</td>
<td>1.25 ± 0.16</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>R = 0.256; NS</td>
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<tr>
<td>Lining thickness (mean)</td>
<td>1.94 ± 0.31</td>
<td>1.92 ± 0.06</td>
<td>1.43 ± 0.19</td>
<td>NS</td>
<td>NS</td>
<td>R = 0.467; p &lt; 0.05</td>
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<td>Synovial lining Mcl-1</td>
<td>83.55 ± 6.82</td>
<td>81.14 ± 0.33</td>
<td>72.85 ± 8.09</td>
<td>NS</td>
<td>NS</td>
<td>R = 0.631; p &lt; 0.001</td>
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<td>staining (% of positive)</td>
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<td>McI-1 Lining staining</td>
<td>2.68 ± 0.12</td>
<td>2.78 ± 0.19</td>
<td>2.14 ± 0.26</td>
<td>NS</td>
<td>NS</td>
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<td>Sublining fibroblast Mcl-1</td>
<td>62.72 ± 3.52</td>
<td>38.33 ± 8.52</td>
<td>27.88 ± 11.32</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.01</td>
<td>R = 0.631; p &lt; 0.001</td>
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<td>staining (% of positive)</td>
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**FIGURE 2.** Expression of Mcl-1 is higher in RA synovial fibroblasts, compared with OA synovial fibroblasts. A, Cell lysates from RA and OA synovial fibroblasts were used to perform Western blot assay to detect Mcl-1. All the RA and OA fibroblasts used were from passages 3–9. The results shown are representative of those obtained from independent synovial fibroblast cultures from four patients with RA and three patients with OA. B, The intensity of the bands identifying Mcl-1 in A was determined by densitometry and normalized with the intensity of tubulin. C, Intracellular staining of Mcl-1 by flow cytometry was performed using synovial fibroblast cultures from an additional four patients with RA and three patients with OA. B and C, The results presented represent the mean ± 1 SE of the relative expression of Mcl-1 in the synovial fibroblast cultures. D, RA synovial fibroblasts from passages 3–9 were treated with TNF-α (10 ng/ml) and IL-1β (10 ng/ml) for 8 h. The cells were harvested, and cell lysates were used to perform Western blot assay using anti-Mcl-1 Abs. E, RA or OA synovial fibroblasts were incubated with control medium (control), TNFα, or IL-1β for 2–4 h. The cells were harvested, and mRNA expression was determined by real-time PCR using specific human Mcl-1 and GAPDH primers. The results are presented as the fold increase of Mcl-1 mRNA relative to GAPDH. The results presented in E represent the mean ± SE of four independent experiments. *, p < 0.05; **, p < 0.01 of the RA, compared with the OA synovial fibroblasts. #, p < 0.02 for RA and <0.01 for OA, compared with control medium.
Bax or Bak in RA synovial fibroblasts (Fig. 6A). Apoptosis, determined by annexin V staining, induced by the reduction of Mcl-1 was significantly suppressed by the reduction of Bax (p < 0.01) or Bak (p < 0.05) (Fig. 6B). These observations suggest that the effect of the reduction of Mcl-1 in RA synovial fibroblasts was mediated through Bax and Bak. Interestingly, the use of siRNA with both Bax and Bak together (Fig. 6A) did not result in reduced apoptosis, compared with reduction with either alone, when apoptosis was induced by the down-regulation of Mcl-1 in RA synovial fibroblasts (Fig. 6B).

The BH3-only molecule, Bim, is capable of activating Bax or Bak and is also capable of binding to Mcl-1 (40, 41). Further studies were performed to determine the potential role of Bim in the apoptosis of RA synovial fibroblasts induced by down-regulation of Mcl-1. Depletion of Bim by Bim siRNA significantly (p < 0.01) suppressed the apoptosis induced by down-regulation of Mcl-1 in RA synovial fibroblasts (Fig. 6C). These observations demonstrate that, in addition to Bax and Bak, Bim is important in the apoptosis induced in RA synovial fibroblasts following the reduction of Mcl-1.

Discussion

This study demonstrates that Mcl-1 is highly expressed in the RA joint and that the expression of Mcl-1 is increased in RA, compared with OA or AFC synovial fibroblasts. The expression of Mcl-1 in sublining synovial fibroblasts correlated with the inflammatory score and TNF-α and IL-1β, inflammatory cytokines important in the pathogenesis of RA, increased the expression of Mcl-1 in RA synovial fibroblasts. Further supporting the role of inflammation, Mcl-1 may be rapidly induced by other cytokines and chemokines, including GM-CSF and IL-6 (42, 43), which are highly expressed in the rheumatoid joint. Together, these observations suggest that Mcl-1 may be important in the persistence or progression of RA. Our study provides novel insights into the mechanisms by which Mcl-1 promotes survival by protecting mitochondrial integrity. The forced reduction of Mcl-1 resulted in the loss of ∆ψm, the release of cytochrome c, the activation of caspase 9 and caspase 3, and subsequent apoptotic cell death in the absence of an additional death signal. The reduction of Mcl-1 in RA synovial fibroblasts resulted in the mitochondrial localization of Bax, and the siRNA-mediated reduction of Mcl-1 alone, in the absence of an additional signal, was not sufficient to induce apoptosis, because Bcl-xL translocated from the cytosol to the mitochondria following the reduction of Mcl-1, preventing the activation of Bak (24, 25, 45). In contrast, in RA synovial fibroblasts, the...
forced reduction of Mcl-1, using Ad-delivered Mcl-1 AS, resulted in apoptotic cell death that was mediated, in part, through Bak.

There are a number of potential explanations for the differences observed. First, the Ad delivered Mcl-1 AS rapidly suppressed the production of Mcl-1, resulting in apoptosis by 24–48 h. It is possible that our Mcl-1 AS was more effective than siRNA at rapidly suppressing the synthesis of Mcl-1. Because the half-life of Mcl-1 is short, ~40 min (25), the rate of suppression of protein synthesis may be critical. Bak was bound to both Mcl-1 and Bcl-xL in healthy cells, and Bcl-xL increased in mitochondria following the reduction of Mcl-1, using siRNA (25, 45). These observations suggest that, if Mcl-1 is more gradually reduced, perhaps using siRNA, cytosolic Bcl-xL is capable of translocating to the mitochondria, preventing the activation of mitochondrial Bak. In the RA synovial fibroblasts, Bcl-xL was enriched in the mitochondria, and there was only a small increase in mitochondrial Bcl-xL following the reduction of Mcl-1 (data not shown). Nonetheless, consistent with the ability of Bcl-xL to substitute for Mcl-1, the ectopic expression of Bcl-xL was as effective as Mcl-1 in the protection of RA synovial fibroblasts against apoptosis induced by the forced reduction of Mcl-1 (Fig. 3E).

Proapoptotic BH3 molecules, such as Bad, Puma, and Noxa, work by binding to and de-repressing the effects of multidomain antiapoptotic molecules, such as Bcl-xL and Mcl-1 (40, 41). It is possible that, in RA synovial fibroblasts, BH3-only proteins such as Bad or Puma may be increased, binding to and inactivating Bcl-xL (40, 45), which is readily detected in RA synovial fibroblasts (data not shown). Additionally, even though the control Ad vector did not induce apoptosis, our data do not exclude the possibility that the infection with the AdMcl-1 AS may have provided an additional signal that, together with the reduction of Mcl-1, induced apoptosis. However, supporting the current observations, the use of AS oligonucleotides to reduce Mcl-1 in macrophages, in the absence of an Ad infection, also induced apoptosis in normal, in vitro-differentiated macrophages (17). Likewise, the reduction of Mcl-1, in the absence of an additional death-inducing signal, also resulted in apoptosis using a multiple myeloma cell line and breast cancer cells (46, 47). These observations suggest that the ability of the forced reduction of Mcl-1 to induce apoptosis, in the absence of an additional death-inducing signal, may be determined by the method of Mcl-1 reduction, the cell type examined, and the disease context.

Our study also documents an important role for Bax in the apoptosis of RA synovial fibroblasts, because Bax translocated to the mitochondria and the reduction of Bax protected the cells from apoptosis following the forced reduction of Mcl-1. The mechanism by which the reduction of Mcl-1 induced the mitochondrial localization of Bax has not been fully elucidated. Even though Mcl-1 is capable of binding with Bax (48), in healthy cells, cytosolic Bax is monomeric and does not appear to bind to other molecules (49), and mitochondrial Bax was not immunoprecipitated with Mcl-1 or Bcl-xL (45). These observations suggest that the direct interaction between Mcl-1 and Bax was not responsible for preventing Bax mitochondrial translocation and apoptosis.

The combination of Bax and Bak siRNA did not result in the enhanced suppression of the cell death, compared with either alone. These observations suggest that both Bax and Bak were capable of mediating the induction of apoptosis following the forced reduction of Mcl-1, and that they may functionally cooperate. Consistent with this interpretation, previous findings demonstrated that, following hypoxia or ATP depletion, Bax translocated from the cytosol to the mitochondria and functionally cooperated with Bak (50). If the effects of Bax and Bak observed in our study were independent, additive protection by Bax and Bak siRNA might have been expected. If either was fully sufficient to mediate apoptosis, as observed in mouse embryonic fibroblasts genetically deficient in Bax and/or Bak (51), the reduction of either Bax or Bak alone would not have protected against apoptosis induced by the reduction of Mcl-1. In mouse embryonic fibroblasts...
deficient in both Bax and Bak, sensitivity to UV-induced apoptosis, which is mediated through Mcl-1, was restored to a greater degree with Bak than Bax, suggesting that, in this cell type, both Bax and Bak contribute, although Bak was more important (45). Our observations demonstrate that Bax and Bak each contribute to the induction of apoptotic cell death in RA synovial fibroblasts following the forced reduction of Mcl-1.

In healthy cells, Bax exists in a latent or inactive form in the cytosol and requires activation to induce mitochondrial localization and the induction of apoptosis (41, 49). Our data suggest that Bim may be important in the activation and movement of Bax, because the siRNA-mediated reduction of Bim protected against the induction of apoptosis following the forced reduction of Mcl-1. Because Bim has been shown to interact with endogenous Mcl-1 in living cells (20), the reduction of Mcl-1 may free Bim, which may then activate Bax (52). Peptides representing the BH3 domain of Bim activated Bax directly (41). In RA synovial fibroblasts, the m.w. of Bim indicates that the major version of Bim is the extra-long isoform (BimEL). Mcl-1 binds to BimEL (53), and when ectopically expressed, BimEL induced apoptosis (54, 55). These observations suggest that following the forced reduction of Mcl-1, Bim may be freed to activate Bax. In RA synovial fibroblasts, additional experiments are needed to directly document the role of Bim in the activation and mitochondrial localization of Bax.

It is also possible that the effect of Bim may be mediated through Bak. In certain cell types, Bim is sequestered to the dynein motor complex. However, in healthy T cells, Bim localized to the mitochondria and bound to Bcl-2 and Bcl-xL, but not apoptosis executioners Bax and Bak (56). Bim also is capable of binding Mcl-1 (40, 53, 56), which also localizes to the mitochondria. Therefore, it is possible that the forced reduction of Mcl-1 resulted in the release of Bim at the level of the mitochondria, permitting the activation of Bak. Supporting the possibility that the effects of Bim may be mediated through both Bax and Bak, recent observations demonstrated that the combined loss of Bak and Bim or Bax and Bim resulted in thymocytes that were markedly more resistant to apoptosis, compared with cells isolated from single knockout mice (57). In summary, our data demonstrate that Mcl-1 is highly expressed in RA synovial fibroblasts and that this may contribute to their resistance to apoptosis-mediated by pathways involving Bim, Bax, and Bak. These data suggest that Mcl-1 may be a powerful therapeutic target to suppress the chronic inflammation observed in the RA joint.

FIGURE 5. Bax translocates to the mitochondria following the reduction of Mcl-1 in RA synovial fibroblasts. A, RA synovial fibroblasts were infected with Ad vectors expressing Mcl-1 AS (AdMcl AS) or the control βgal (Adβgal) at 100 moi and then cultured for 6–18 h. Cells were harvested, and cytosolic fraction and the heavy membrane fraction enriched in mitochondria were isolated and used for Western blot analysis using anti-Bax Abs. B, RA synovial fibroblasts were infected with AdMcl AS or the control Adβgal at 100 moi and then cultured for 16 h. The cells were then incubated with Mitotracker Red (50 nM) for 30 min to identify the mitochondria. The cells were fixed, permeabilized, stained with Bax, and examined by confocal microscopy as described in Materials and Methods. The results presented are representative of three independent experiments.

FIGURE 6. Depletion of Bax, Bak, or Bim protects against apoptosis induced by down-regulation of Mcl-1 in RA synovial fibroblasts. RA synovial fibroblasts were transfected with Bax, Bak (A and B), Bim (C), or control siRNA (A–C) for 72 h. Following infection with AdMcl-1 AS at 200 moi, the cells were cultured for 24 h and then harvested for Western blot analysis to detect Bax and Bak (A) or Bim (C), or to document apoptosis using annexin V staining (B and C). *, p < 0.05; **, p < 0.01 between control and Bax, Bak, or Bim siRNA. The results presented are representative of three independent experiments.
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Disclosures

The authors have no financial conflict of interest.

References


