Central role of mitochondria and p53 in Fas-mediated apoptosis of rheumatoid synovial fibroblasts

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Objective. Fas-mediated apoptosis is preferentially observed in synoviocytes of patients with rheumatoid arthritis (RA) and is associated with the pathophysiological process of RA. To clarify the molecular mechanisms of Fas-mediated apoptosis of RA synoviocytes, we investigated the role of the mitochondrial pathway and tumour suppressor p53 in this process.

Methods. Cultured synovial fibroblasts were prepared from RA patients. After treatment of RA synovial fibroblasts with anti-Fas monoclonal antibody, the expression levels of activated caspase-9 and -3, Bid cleavage, cytochrome c release and phosphorylation of p53 at Ser15 were assessed using immunoblot analysis. The mitochondrial membrane potential (ΔΨm) was evaluated with a fluorescence-based detection assay. Apoptotic cells were determined by a DNA fragmentation assay in the presence or absence of caspase inhibitors. Expression of p53-regulated apoptosis-inducing protein 1 (p53AIP1) was measured by real-time PCR. RA synovial fibroblasts stably transfected with a dominant-negative (DN) p53 were prepared in order to investigate the role of p53 during Fas-induced apoptosis.

Results. Fas ligation induced Bid cleavage, loss of ΔΨm, cytochrome c release to the cytosol and activation of caspase-9 and -3 in RA synovial fibroblasts. Treatment with a caspase-9-specific inhibitor almost completely inhibited Fas-mediated apoptosis. Moreover, p53 activation after Fas ligation was evidenced by its phosphorylation at Ser15 and up-regulation of the p53 target gene p53AIP1. Fas-mediated apoptosis was significantly suppressed by anti-sense p53 oligonucleotides and by p53DN.

Conclusion. Our findings strongly suggest the involvement of mitochondria and p53 in Fas-mediated apoptosis of RA synovial fibroblasts.

Key words: Rheumatoid arthritis, Synoviocyte, Apoptosis, Fas, Mitochondria, Caspase-3, Caspase-9, Bid, p53, p53AIP1.

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by abnormal synovial hyperplasia associated with local infiltration of various inflammatory cells, which leads to cartilage and bone destruction in the inflamed joints [1]. However, synovial proliferation is limited and spontaneous regression is occasionally observed during the course of the disease [2, 3]. Apoptosis, particularly mediated by Fas (CD95/APO-1), is closely associated with the pathophysiology of RA [4]. We and other investigators reported previously that synoviocytes in the RA synovium expressed functional Fas antigen and that these cells were sensitive to Fas-mediated apoptosis both in vitro and in vivo, in contrast to osteoarthritis (OA) synoviocytes [5–7]. However, intractable synovial hyperplasia is often observed during the course of RA, suggesting that Fas-mediated apoptosis may be incapable of fully eliminating cells in the proliferative RA synovium. Elimination of proliferating RA synoviocytes by regulating the apoptotic processes might therefore be a potentially effective treatment modality for RA [8–11]. Thus, clarification of the precise signalling events in Fas-mediated apoptosis of RA synoviocytes is important, but these events are poorly understood.

In Fas-mediated apoptosis, two different signalling pathways have been identified in a variety of cell types [12]. The type I pathway requires activation of caspase-8 at the death-inducing signalling complex (DISC), closely followed by activation of caspase-3. In the type II pathway, caspase-3 is activated by caspase-9 via activation of Bid by caspase-8, loss of mitochondrial membrane potential (ΔΨm) and cytochrome c release to the cytosol. This apoptotic pathway is also called the mitochondrial pathway. In cells expressing the type II pathway, apoptosis is blocked by overexpression of Bcl-2 or Bcl-XL, whereas this effect is not observed in type I pathway-controlled cells [13, 14]. In our previous studies, we demonstrated the crucial involvement of the DISC formation and caspase-3 activation in Fas-mediated apoptosis of RA synovial fibroblasts [15–18]. However, we have not yet determined whether Fas-mediated apoptosis of RA synovial fibroblasts is mediated by the type I or the type II pathway.

In the mitochondrial apoptotic pathway, p53 plays an important role in mitochondrial membrane stability and promotes apoptosis [19, 20]. p53 is a tightly regulated transcription factor that induces cell cycle arrest or apoptosis in response to cellular stress, such as DNA damage [21]. It is now clear that a number of phosphorylation sites on p53 are altered after DNA damage, and such phosphorylation events have been shown to result in alterations in p53 that make it more stable and more active [20, 22]. An important p53 transcription target gene was identified as p53-regulated apoptosis-inducing protein 1 (p53AIP1), which is located in the mitochondrial membrane and is directly involved in p53-dependent mitochondrial apoptosis [23, 24]. High levels of p53 expression have been observed in the RA synovium [25–27]. While inactivating somatic mutations of p53 were found in RA synovial fibroblasts [28–31], providing an explanation for the tumour-like growth of RA synoviocytes, other studies reported a lack of p53...
gene mutations and that the endogenous p53 gene was functionally active [32, 33]. Thus, whether p53 in RA synovium was wild-type or mutated was controversial [34]. Recently, however, Yamanishi et al. [35], using microdissection analysis, confirmed that only a limited number of RA synoviocytes contained p53 mutations.

The present study was designed to examine the association between p53 and Fas-mediated apoptosis of RA synoviocytes. For this purpose, we investigated the involvement of the type II pathway and p53 in Fas-mediated apoptosis of RA synovial fibroblasts. We found that the mitochondrial apoptotic pathway played a central role in Fas-mediated apoptosis of RA synovial fibroblasts and that p53 was secondarily activated to amplify the apoptotic cascade at the mitochondrial level.

Materials and methods

Antibodies

The following antibodies were used in this study: caspase-3, cytochrome c, poly(ADP-ribose) polymerase (PARP), Bcl-XL, p53, FITC (fluorescein isothiocyanate)-labelled Fas (CD95) (BD Pharmingen, San Diego, CA, USA); caspase-9, phospho-p53 (Ser15) (Cell Signaling Technology, Beverly, MA, USA); Bid (Biosource International, Camarillo, CA, USA); Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Bax, caspase-8 (Medical & Biological Laboratories, Nagoya, Japan); Fas (clone-7C11, Immunotech, Marseille, France); and control murine IgM antibody, β-actin (Sigma-Aldrich, St Louis, MO, USA).

Cell cultures and treatment conditions

Patients with RA were diagnosed according to the criteria of the American College of Rheumatology. Following informed consent, synovial tissues were obtained from four patients with RA and three patients with OA undergoing total joint replacement. Cultured synovial fibroblast cell lines were established as described previously [36]. Cells were cultured in Ham’s F-12 (Sigma-Aldrich) supplemented with 1 mM l-glutamine, 1 mM sodium pyruvate, 5.5 x 10^{-2} M 2-mercaptoethanol, 100 U/ml penicillin, and 10% fetal calf serum (FCS) (all from Invitrogen, Carlsbad, CA, USA). Cultured RA and OA synovial fibroblasts were used at passages 5–10 in all experiments. To enhance Fas-mediated apoptosis, synovial fibroblasts were pretreated with tumour necrosis factor α (TNF-α) (PeproTech, Rocky Hill, NJ, USA) at a concentration of 5 ng/ml for 5 days [16, 18]. Synovial fibroblasts were seeded in 96-well plates at 5 x 10^{5} cells/well or 60 mm dish plates at 1 x 10^{6} cells/plate. After 24 h, the medium containing TNF-α was removed and fresh medium containing anti-Fas monoclonal antibody (mAb) (7C11) at a concentration of 1.0 μg/ml was added to the cells. For treatment with caspase inhibitors, caspase inhibitors [zVAD-fmk, zLEHD-fmk (Calbiochem, La Jolla, CA, USA) or Ac-YVD-CHO (Peptide Institute, Osaka, Japan)] or the carrier DMSO was added 30 min before Fas ligation with anti-Fas mAb.

DNA fragmentation assay

The DNA fragmentation assay was performed using cell-death detection ELISA (enzyme-linked immunosorbent assay) Plus (Roche Diagnostics, Penzberg, Germany) according to the manufacturer’s instructions. Briefly, after treatment with anti-Fas mAb in the presence or absence of caspase inhibitors, cytoplasmic histone-associated DNA fragments released into the culture supernatant were measured by a quantitative sandwich enzyme immunoassay using mouse mAb directed against DNA and histones. Absorption values at 405 nm were determined with an automatic microtitre plate reader (Bio-Rad Laboratories, Hercules, CA, USA). The DNA fragmentation rate (%) was calculated using the formula [(OD_{405II} – OD_{405I})/(OD_{405III} – OD_{405I})] x 100, where OD_{405I} is optical density (405 nm) with reaction buffer alone, OD_{405II} is optical density (405 nm) with supernatant of treated cells and OD_{405III} is optical density (405 nm) with supernatant of non-treated cells.

Immunoblot analysis

After treatment with anti-Fas mAb, the reaction was terminated by addition of ice-cold phosphate-buffered saline (PBS). As described in our previous study [37], cell pellets were lysed with 0.5% sodium dodecyl sulphate (SDS) solution and proteins (8–16 μg) were separated by SDS-PAGE (polyacrylamide gel electrophoresis), transferred to an Immobilon-P membrane (Millipore, Billerica, MA, USA) or nitrocellulose membrane (Bio-Rad Laboratories), blocked with 5% skim milk, and immunoblotted with arbitrary antibody and horseradish peroxidase-labelled secondary antibody. The blots were developed using the enhanced chemiluminescent substrate (Super Signal West Pico; Pierce Chemical, Rockford, IL, USA) and visualized with a LumiVision analyser (Taitec, Tokyo, Japan). Densitometric analysis was performed with a LumiVision analyser (Taitec).

Cytochrome c release assay

After treatment with anti-Fas mAb, the reaction was terminated by addition of ice-cold PBS. As described in our previous report [37], cell pellets were lysed in isotonic buffer A containing 0.1 mM digitonin (Sigma-Aldrich) plus protease inhibitors (0.2 mM phenylmethylsulphonyl fluoride, 20 μg/ml aprotinin, 10 μg/ml pepstatin A and 10 μg/ml leupeptin). After incubation on ice for 5 min, the cytosolic fraction was collected and the insoluble fraction was disrupted by sonication. The amount of cytochrome c released to the cytosol was evaluated by immunoblot analysis using anti-cytochrome c mAb as described above.

Measurement of mitochondrial membrane potential (ΔΨm)

After treatment with anti-Fas mAb in the presence or absence of caspase inhibitors, cells were harvested with trypsin–EDTA solution (Sigma-Aldrich) and resuspended in PBS containing 10 μM EDTA. Then, the cells were subjected to flow cytometric analysis. The changes in ΔΨm were tested using the Mitolight apoptosis detection kit (Chemicon International, Temecula, CA, USA) according to manufacturer’s instructions. Labelled cells were analysed using a fluorescence-activated cell sorting (FACSCalibur; Becton Dickinson, San Jose, CA, USA).

Real-time PCR

The expression of p53AIP1 [23] was determined by real-time quantitative reverse transcription–polymerase chain reaction (PCR) according to our previous report [37]. Briefly, total RNA was extracted, reverse-transcribed into cDNA, and amplified by real-time quantitative PCR for p53AIP1. The sense and antisense primers for p53AIP1 were 5′-GATCTTCTCTGAGGCGAGCT-3′ and 5′-AAGACCGAGCATGTTGTT-3′, respectively. The sense and antisense primers for β-actin were 5′-TTACATCAGTGGCCATCTATCTAGA-3′ and 5′-CACGGAAAGTCGCTTCTCCGCG-3′, respectively. The reaction master mix (PE Applied Biosystems) containing the p53AIP1 hybridization probe (5′-CAGATCTGCTCAAGCTTTCTG-3′) labelled with 6-carboxyfluorescein at the 5′ end was used. β-Actin
from the predeveloped TaqMan Assay Reagents (PE Applied Biosystems) was used as endogenous control. Input RNA amounts were calculated with relative standard curves for mRNA of p53AIP1 and β-actin. The transcript abundance of p53AIP1 was calculated as the ratio of experimental sample values normalized to β-actin RNA expression.

**p53 antisense oligodeoxynucleotide and transfection protocol**

Phosphorothioate p53 antisense oligonucleotide (p53 AS) (5'-CCCTGCTCCCCCTGCTCC-3') and scrambled control oligonucleotides (5'-CGGTGATCTCCAGATGTGC-3'), used as negative controls [38], were obtained from Invitrogen. Transfection was performed using cationic lipopolymamines. Synovial cells were seeded in 96-well plates at 1 x 10^4 cells/well. After 24 h, DNA–lipopolyamine complex containing 1 μl/ml Oligofectamine reagent and p53AS or scrambled control oligonucleotides in Opti-MEM was added at a final concentration of 100 μM DNA (all from Invitrogen). After 72 h, cells were washed and used for the experiment.

**Establishment of RA synovial fibroblasts expressing dominant-negative p53**

A p53 dominant-negative (p53DN) expression vector designated pBCMGS-p53DN was used, in which cysteine at position 135 in the p53-binding site is mutated to tyrosine (C135Y) [37]. Briefly, a cDNA that encoded full-length p53DN was subcloned from pCMV-p53mt135 vector (Clontech) into pBluescript SK(+), (Stratagene, La Jolla, CA, USA) and finally inserted into the pBCMGSneo expression vector. Synovial cells were transfected with pBCMGS-p53DN using cationic lipopolymamines. Briefly, synovial fibroblasts were seeded in six-well plates at 1 x 10^5 cells/well. After 24 h, premixture containing Lipofectamine reagent (Invitrogen) with the pBCMGS-p53DN or pBCMGSneo in Opti-MEM was added at a final concentration of 1 μg DNA/ml. After 4 h, transfected cells were washed and subjected to G418 selection to establish stable transfectants.

**Statistical analysis**

All data are expressed as mean ± S.D. Differences between groups were examined for statistical significance using Student's t-test. A P value of less than 0.05 denoted a statistically significant difference.

**Results**

**Mitochondrial pathway in Fas-mediated apoptosis of RA synovial fibroblasts**

In this study, we used cultured RA synovial fibroblast lines with constitutive expression of Fas (CD95/APO-1) on the cell surface [4], and anti-Fas monoclonal antibody (IgM) was added to induce Fas-mediated apoptosis. The ratio of apoptotic cells was evaluated by DNA fragmentation assay after Fas ligation. As shown in Fig. 1A, apoptotic cells started to appear at 6 h, constituted approximately 15% of the cells at 9 h and approximately 40% at 24 h. On the other hand, cultured OA synovial fibroblasts showed no significant apoptosis after Fas ligation. The apoptotic cells were spherical in shape and detached from the bottom of the culture plates, whereas no marked changes were observed in synovial fibroblasts treated with control mouse IgM antibody (data not shown). To evaluate the involvement of the mitochondrial pathway in the above Fas-mediated apoptosis of RA synovial fibroblasts expressing dominant-negative p53, the expression levels of Bid, caspase-9, caspase-3, pro-caspase-3, PARP, and β-actin were analyzed by immunoblotting. As shown in Fig. 1B, the expression levels of Bid, caspase-9, and caspase-3 were significantly increased after Fas ligation. However, the expression levels of pro-caspase-3, PARP, and β-actin were not significantly changed. The results suggest that the mitochondrial pathway is involved in Fas-mediated apoptosis of RA synovial fibroblasts expressing dominant-negative p53.

**Fig. 1.** Activation of Bid and caspase-9 during Fas-mediated apoptosis in RA synovial fibroblasts. (A) After treatment of cultured RA and OA synovial fibroblasts with anti-Fas mAb or control antibody (1.0 μg/ml) for the indicated times, the apoptosis rate (%) was measured by DNA fragmentation assay. The values are reported as mean and S.D. of triplicate wells. (B) Cultured RA synovial fibroblasts were treated with anti-Fas mAb or control antibody for the indicated time and subjected to immunoblot analysis as described in Materials and methods. β-Actin was detected as a loading control. (C) Histogram representing relative expression of the indicated proteins analysed in panel B. Band density is expressed as the percentage of the maximum level of each protein at the indicated time after Fas ligation. Data shown (mean and s.d.) are representative of three experiments. *P < 0.01.
fibroblasts, we examined the cleavage patterns of Bid, caspase-9, caspase-3 and PARP by immunoblotting. Activated caspase-8 is known to cleave p22 Bid to its active form, p15 truncated Bid (tBid), which translocates to the mitochondrial membrane to form a complex with Bcl-XL, which in turn causes a decrease in ΔΨm, and the compartment consisting of cytochrome c released to the cytosol forms a complex with Apoptosis protease-activating factor 1 (Apaf1) and activates caspase-9 [39–42]. As shown in Fig. 1B and C, cleavage of caspase-9, caspase-3 and PARP was clearly observed after Fas ligation for 6 h, and Bid was initially cleaved to form a 15 kDa fragment at 3 h (*P < 0.01). Cytosolic extracts prepared from Fas-ligated synovial fibroblasts revealed the release of cytochrome c from the intramitochondrial space into the cytosol (Fig. 2A and B). In agreement with this finding, Fas ligation caused marked dissipation of ΔΨm (Fig. 2C). Immunoblotting analysis of Bcl-2 family members revealed a significant reduction of the anti-apoptotic protein Bcl-XL at 6 and 9 h (*P < 0.05 and **P < 0.01 respectively), whereas the anti-apoptotic molecule Bcl-2 was induced at 3 h and expression of the pro-apoptotic molecule Bax was unchanged (Fig. 2D and E). These results clearly indicate that Fas ligation induces activation of the mitochondrial apoptotic pathway through tBid production and alteration of the balance among Bcl-2 family members in RA synovial fibroblasts.

Requirement of caspase-9 in Fas-mediated apoptosis of RA synovial fibroblasts

To further explore the direct involvement of the mitochondrial pathway via activation of caspase-9 during Fas-mediated apoptosis, RA synovial fibroblasts were cultured with or without anti-Fas antibody, in the presence or absence of serial dilutions of the caspase-9-inhibitory peptide zLEHD-fmk, and apoptosis was evaluated by DNA fragmentation assay. As shown in Fig. 3A, zLEHD-fmk suppressed Fas-mediated apoptosis in a dose-dependent manner, and completely inhibited apoptosis at a concentration of 50 μM (*P < 0.01). To ensure specificity for caspase-9, we confirmed that zLEHD-fmk at a concentration of 50 μM had no effect on the activation of caspase-8 induced by Fas ligation (Fig. 3B and C). On the other hand, zVAD-fmk, a broad-spectrum caspase inhibitor, completely inhibited Fas-mediated apoptosis at a concentration as low as 12.5 μM, which had no effect on the activation of caspase-8 induced by Fas ligation (data not shown), whereas Ac-YVAD-CHO, a compound known to inhibit caspase 1-like protease activity [16] and used here as a specificity control, had no effect.

Next, we examined the importance of caspase-9 for activation of caspase-3, a final executor of apoptosis. For this purpose,
we evaluated caspase-3 activity in the presence of zLEHD-fmk after Fas ligation. As shown in Fig. 3D and E, zLEHD-fmk, as well as zVAD-fmk, significantly inhibited the activation of caspase-3 ($P < 0.05$ and $P < 0.01$ respectively). These results clearly indicate that activation of caspase-3 is required for initiation of the caspase cascade downstream from mitochondria and that the mitochondria-dependent pathway is dominant in Fas-mediated apoptosis of RA synovial fibroblasts.

**p53 activation in Fas-mediated apoptosis of RA synovial fibroblasts**

On the basis of our finding of a central role for the mitochondrial apoptotic pathway in RA synovial fibroblasts, we next examined the involvement of p53 in this process. To identify possible alterations in the function of p53 in response to Fas ligation in RA synovial fibroblasts, we examined p53 phosphorylation at Ser15, which reflects its functional response to cellular stress, such as DNA damage, and leads to apoptosis [20, 22]. We found that Fas ligation induced p53 phosphorylation after 6 h (Fig. 4A and B), and this was accompanied by transcriptional activation of p53AIP1 (Fig. 4C). These results indicate that Fas ligation induces p53 activation and subsequently up-regulates p53AIP1 expression in RA synovial fibroblasts, which is relevant to the direct induction of mitochondria-dependent apoptosis [23]. Importantly, zVAD-fmk almost completely abrogated the induction of p53AIP1 ($P < 0.05$) (Fig. 4C) and phosphorylation of p53 ($P < 0.01$) (Fig. 4D and E) induced by Fas ligation. These results suggest that p53 activation in RA synovial fibroblasts occurs downstream of caspase-3 after Fas ligation rather than p53 activation being primarily induced by Fas ligation.

**Involvement of both p53-independent and -dependent pathways in Fas-mediated apoptosis of RA synovial fibroblasts**

To confirm that p53 is indeed responsible for the promotion of Fas-mediated apoptosis in RA synovial fibroblasts, we examined whether Fas stimulation can induce apoptosis in cells treated with...
p53 antisense oligonucleotides. Despite Fas ligation, blockade of p53 by antisense oligonucleotides significantly inhibited Fas-mediated apoptosis at a concentration of 200 M (P < 0.05) (Fig. 5A). No significant difference was observed between control oligonucleotide-transfected and -untransfected cells in the DNA fragmentation assay, and the blocking effect of p53 antisense oligonucleotides was observed in all RA synovial fibroblast cell lines tested (data not shown). Finally, to confirm the notion that p53 activation is secondarily induced in response to cellular stress, such as DNA damage by caspase-3 activation, and to analyse the extent of p53 involvement in Fas-mediated apoptosis of RA synovial fibroblasts, we established RA synovial fibroblasts that were stably transfected with a dominant-negative form of p53 (p53DN) in order to completely abrogate endogenous p53 function. p53DN-transfected RA synovial fibroblasts exhibited significant attenuation of Fas-mediated apoptosis (P < 0.05), but still showed a substantial level of apoptosis compared with the vector control (Fig. 5B). These p53DN RA synovial fibroblasts showed that p53AIP1 expression after Fas ligation was almost completely suppressed to the basal level compared with p53DN in the presence of control antibody (P < 0.05) (Fig. 5C). Importantly, zLEHD-fmk further inhibited Fas-mediated apoptosis of p53DN RA synovial fibroblasts (P < 0.05) (Fig. 5B). These results strongly suggest that Fas ligation primarily activates the p53-independent apoptotic pathway and then activates the p53-dependent apoptotic pathway, resulting in p53-amplified apoptosis of RA synovial fibroblasts.

**Discussion**

Studies by our group and others have independently demonstrated the expression of functional Fas antigen on RA synoviocytes and have shown that these cells undergo Fas-mediated apoptosis both in vivo and in vitro [5–7]. Using two different models of RA [human T-cell leukaemia virus type I-carrying mice and mice with severe combined immunodeficiency (SCID) engrafted with human RA synovium], we have previously demonstrated the effectiveness of active induction of Fas-mediated apoptosis in producing marked regression of proliferative synovium [8–10]. Taken together, these findings suggest that the Fas system present in RA synovium may prevent synovial hyperplasia. In RA synovium, synovial macrophages primarily play important roles in synovial fibroblast proliferation and chronic inflammation by producing TNF-α and interleukin 1 [11]. In addition, TNF-α sensitizes synovial fibroblasts for Fas-mediated apoptosis at least in part by up-regulation of caspase-8 and caspase-3, and down-regulation of FADD-like interleukin-1β-converting enzyme (FLICE)-like inhibitory protein (FLIP) [11, 16, 18], whereas it has been suggested that TNF-α might induce FLIP expression in synovial macrophages [11]. This
indicates that TNF-α is involved in Fas-mediated apoptosis as well as cell proliferation of RA synovial fibroblasts in vivo. In the present study, we pretreated synovial fibroblasts with TNF-α to enhance their Fas-mediated apoptosis. Accordingly, the mechanisms of Fas-mediated apoptosis in synovial macrophages as well as synovial fibroblasts need to be clarified, and synovial macrophages will be potentially crucial targets for therapeutic intervention in RA. Recently, it has been reported that apoptosis is a rare event in recent-onset RA but is a more frequent event in late disease, and that apoptosis levels in the RA synovium correlate negatively with FLIP expression and synovial macrophage score [43]. This suggests that active induction of Fas-mediated apoptosis as a treatment for RA may be efficacious in the late stage rather than in the early stage, because FLIP regulates Fas-mediated signalling by inhibiting DISC formation. Thus, it is important to understand the precise molecular mechanisms of Fas-mediated apoptosis in RA synoviocytes in order to facilitate its clinical application to RA treatment.

To date, the molecular mechanisms of Fas-mediated apoptosis regulated by DISC formation, FLIP expression, caspase-3 activation and cytokines have been described in RA synovial fibroblasts [15–18]. However, the complete mechanism of Fas-mediated apoptosis of RA synovial fibroblasts, especially the involvement of mitochondria and p53, which are major players in apoptosis, has not been elucidated. In the present study, we demonstrated that Fas ligation resulted in cleavage of Bid, loss of mitochondrial membrane potential, release of cytochrome c, caspase-9 activation and caspase-3 activation (Figs 1 and 2), suggesting that the type II mitochondrial apoptotic pathway is essential during Fas-induced apoptosis in RA synovial fibroblasts. This notion is confirmed by our findings that the final apoptosis events, such as DNA fragmentation, were completely inhibited by administration of the caspase-9-specific inhibitor zLEHD-fmk (Fig. 3). In the type II pathway of Fas-mediated apoptosis, the cascade is initiated by the cleavage of Bid by activated caspase-8 [40]. Our previous [15–18] and present (Fig. 1B) studies clearly show that activation of caspase-8 and production of cBid occur in RA synovial fibroblasts immediately after Fas ligation. In Bid-deficient cells from mice resistant to Fas-mediated apoptosis, mitochondrial dysfunction was delayed, cytochrome c was not released, effector caspase activity was reduced and the cleavage of apoptosis substrates was altered [44], indicating its essential role in initiating the type II apoptotic pathway. However, since the cleaved form of caspase-3 was not completely excluded by treatment with zLEHD-fmk, which completely inhibited Fas-mediated apoptosis, as assessed by DNA fragmentation assays (Fig. 3), it is possible that, to some extent, the mitochondria-independent type I apoptotic pathway contributes to Fas-mediated apoptosis of RA synovial fibroblasts.

We identified transactivation of p53 in RA synovial fibroblasts after Fas ligation, as evidenced by its phosphorylation at Ser15 and up-regulation of its target gene p53AIP1 (Fig. 4). This is consistent with the notion of coexistence of apoptosis, Fas and p53 in RA synovial tissue reported by Chou et al. [45], where apoptosis was strongly associated with the expression of Fas and p53, but not Bcl-2. Thus, Fas-induced p53 activation might play a major role in executing the apoptosis of proliferating RA synovial fibroblasts. In accordance with findings in RA synovial fibroblasts, Yin et al. [46] recently reported that Fas-mediated apoptosis is dependent on p53 in some human tumour cell lines. In support of the relevance of p53 to apoptosis in RA synovial cells in vivo, apoptosis in the synovia of p53-deficient was rare in DBA/1 mice with collagen-induced arthritis compared with wild-type mice [47]. Moreover, the loss of p53 function by addition of E6 protein in human synovial cells was associated with increased proliferation and invasion into
cartilage in a SCID mouse model [48]. Thus, these observations support the notion that the p53 gene is expressed in RA in response to inflammation and genotoxic stimulation, as a protective mechanism to induce cell-cycle arrest and apoptosis.

The Fas-induced p53 transactivation observed in RA synovial fibroblasts was dependent on caspase-3 activity, because p53 activation was almost completely inhibited by zVAD-fmk at the low concentration, which had no effect on caspase-8 activation (Fig. 4). In addition, treatment with p53 antisense oligonucleotides and p53DN with no p53A1P1 expression was still associated with a substantial level of Fas-mediated apoptosis in RA synovial fibroblasts, which was completely inhibited by zLEHD-fmk (Fig. 5). ZVAD-fmk treatment of RA synovial fibroblasts and p53DN still induced substantial dissipation of ΔΨm after Fas ligation (data not shown). These results clearly indicate the presence of a p53-independent mitochondrial apoptotic pathway, which is primarily activated by Fas ligation, and which in turn activates a p53-dependent mitochondrial apoptotic pathway in RA synovial fibroblasts. It appears that this p53-amplified loop of mitochondrial apoptosis is not always specific for Fas ligation in RA synovial fibroblasts. Recently, we demonstrated that B-cell receptor-mediated apoptosis of human B cells uses the same mechanism [37]. Thus, it is possible that the p53-amplified loop of the mitochondrial apoptotic pathway is a general mechanism of cell death independent of triggering signals for apoptosis. In addition, because Fas expression is positively regulated by p53 [49–51], it is likely that the mitochondrial apoptotic pathway is further amplified by p53-induced Fas.

On the other hand, activation of c-Jun NH2-terminal kinase (JNK) is induced by UV-C, γ-radiation, DNA-damaging drugs and Fas ligation [52–56]. Activation of the JNK pathway can lead to cell death, in which JNK directly phosphorylates p53 to disrupt MDM2-p53 interaction, prevents p53 degradation and, subsequently, enhances p53-transcriptional activity [57, 58]. Thus, it is assumed that Fas ligation induces p53 activation through JNK instead of a p53-independent pathway. Previously, we also reported that Fas ligation induced rapid tyrosine phosphorylation of JNK and formation of activator protein-1 (AP-1) during apoptosis of RA synovial fibroblasts [15]. However, it is less likely that p53 activated directly by JNK contributes to Fas-mediated apoptosis of RA synovial fibroblasts, because zVAD-fmk completely inhibited p53 activation and apoptosis (Figs 3 and 4), indicating p53 activity downstream of caspase-3, as discussed above.

Taken together, our results clearly indicate that the mitochondria-dependent pathway is dominant and that p53 is involved in the mitochondrial amplification loop in Fas-mediated apoptosis of RA synovial fibroblasts. Novel therapeutic approaches focusing on an additional role for p53 in the mitochondrial apoptotic pathway could be beneficial in RA by helping to restore synovial homeostasis. Indeed, it has been reported that overexpression of p53 by adenoviral infection resulted in massive apoptosis of inflamed synovial tissue in a rabbit model of arthritis [59].

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