Importance of NF-κB in rheumatoid synovial tissues: in situ NF-κB expression and in vitro study using cultured synovial cells

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Abstract

***Objectives*—To examine whether inhibition of NF-κB induces apoptosis of human synovial cells stimulated by tumour necrosis factor α (TNFα), interleukin 1β (IL1β), and anti-Fas monoclonal antibody (mAb).**

***Methods*—The expression of proliferating cell nuclear antigen (PCNA), NF-κB, and the presence of apoptotic synovial cells were determined in synovial tissues. Apoptosis of cultured synovial cells was induced by inhibition of NF-κB nuclear translocation by Z-Leu-Leu-Leu-aldehyde (LLL-CHO). The activation of caspase-3 and expression of XIAP and cIAP2 in synovial cells in LLL-CHO induced apoptosis was also examined.**

***Results*—Abundant PCNA+ synovial cells were found in rheumatoid arthritis (RA) synovial tissue, though a few apoptotic synovial cells were also detected in the RA synovial tissues. Nuclear NF-κB was expressed in RA synovial cells. Electrophoretic mobility shift assay showed that treatment of cells with TNFα or IL1β significantly stimulated nuclear NF-κB activity. A small number of apoptotic synovial cells expressing intracellular active caspase-3 were found after treatment of cells with LLL-CHO. Although treatment of RA synovial cells with TNFα or IL1β alone did not induce apoptosis, apoptosis induced by LLL-CHO and caspase-3 activation were clearly enhanced in TNFα or IL1β stimulated synovial cells compared with unstimulated synovial cells. Furthermore, induction of apoptosis of synovial cells with caspase-3 activation by anti-Fas mAb was clearly increased by LLL-CHO. The expression of cIAP2 and XIAP in synovial cells may not directly influence the sensitivity of synovial cells to apoptosis induced by LLL-CHO.**

***Conclusion*—The results suggest that NF-κB inhibition may be a potentially important therapeutic approach for RA by correcting the imbalance between apoptosis and proliferation of synovial cells in RA synovial tissue.**

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Rheumatoid arthritis (RA) is characterised by pronounced hyperplasia of synovial tissue. Apoptosis of synovial cells in RA synovial tissue is thought to be relatively suppressed compared with the proliferation of the cells, leading to abnormal hyperplasia of synovial cells.[1–4] Cytokines are one of the factors that modulate the balance between cell proliferation and apoptosis.[4 5] Among the cytokines found in the synovial tissue of patients with RA, tumour necrosis factor α (TNFα) and interleukin 1β (IL1β) are thought to have an important role in the development of chronic synovitis in patients with RA.[4–6] Previous studies have shown that both TNFα and IL1β perpetuate the inflammatory process, by stimulating the expression of costimulating molecules on synovial cells.[6]

The mechanism of TNFα and IL1β induced stimulation of the inflammatory process is thought to be mediated by activation of transcriptional factor, NF-κB.[7 8] NF-κB activation, in turn, protects against apoptotic signals,[9–12] suggesting a strong involvement of NF-κB in the regulation of growth of RA synovium.

Nuclear NF-κB expression, which indicates the activation of NF-κB, has been determined in human RA synovium.[13 14] In addition, NF-κB is activated in the synovium of rats with streptococcal cell wall induced arthritis or collagen induced arthritis in rats.[3 15] Furthermore, inhibition of NF-κB activation in this model enhanced apoptosis of synovial cells.[3] Synovial cells treated with TNFα in vitro do not undergo apoptosis,[16 17] indicating that the apoptogenic signals through TNF receptor in synovial cells may be counterbalanced by the concomitant activation of NF-κB. These data strongly support the view that NF-κB is a potential target molecule for the treatment of RA. We show here the importance of NF-κB in the regulation of apoptosis of human synovial cells stimulated by TNFα, IL1β, and anti-Fas monoclonal antibody (mAb).

Patients and methods

PATIENTS

We obtained tissue specimens from patients with RA who met the American College of Rheumatology criteria for RA[18] and patients with osteoarthritis (OA) during orthopaedic surgery in Ureshino national hospital between April 1998 and April 2000. The mean disease duration of patients with RA examined here is 13.4 years (range 3–30). We selected patients with RA who had active inflammation as shown by an increase of C reactive protein (>20 mg/l at the time of orthopaedic surgery). All the patients with RA had received disease modifying antirheumatic drugs—for example, gold

salts, D-penicillamine, sulfasalazine, and bucillamine. About 60% of the patients had a history of steroid treatment, including local injection into the inflamed joints. Non-steroidal anti-inflammatory drugs were used for about 80% of the patients. Informed consent was obtained from all participating subjects and the study was conducted in accordance with the human experimental guidelines of our institution.

MONOCLONAL AND POLYCLONAL ANTIBODIES FOR IMMUNOHISTOCHEMISTRY

We estimated the proliferating cells in situ as proliferating cell nuclear cell antigen positive (PCNA+) cells by the use of mouse antihuman PCNA mAb (Dako A/S, Denmark). Rabbit antihuman NF-κB (p65) polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). We used normal mouse IgGa2 or normal rabbit serum (Dako A/S, Denmark) as a control immunoglobulin for PCNA and NF-κB immunohistochemistry, respectively. Immunohistochemical analysis was carried out by observing 300 cells in the specimen.

IMMUNOHISTOCHEMICAL ANALYSIS OF SYNOVIAL TISSUES

Synovial tissues were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4), and were immersed in 10, 15, and 20% sucrose, successively. Tissues were then frozen in liquid nitrogen and stored at −80°C until use. Synovial tissues used for NF-κB staining were snap frozen and stored until use. Synovial tissue sections (4 μm thick) were cut and mounted on glass slides precoated with aminopropyltriethoxysilane. Dried cryostat sections of snap frozen synovial tissues were fixed by a mixture of methanol and acetone (vol/vol, 1/1) for 10 minutes on ice. Endogenous peroxide activity was inhibited by immersing the section in 3% H_2O_2. After blocking with 10% goat or mouse IgG for 10 minutes, sections were incubated with anti-NF-κB antibody in PBS containing 1% fetal bovine serum (FBS) for 60 minutes in a humidified chamber at room temperature. Sections stained with anti-PCNA antibody were incubated overnight at 4°C. These sections were washed with PBS containing 0.25% Brij and then stained using the streptavidin-biotin method (Histofine staining kit, Nichirei Co, Tokyo). In brief, the sections were treated with biotinylated antimouse IgG or antirabbit IgG for 12 minutes. Sections were washed and then incubated with peroxidase conjugated streptavidin for six minutes in a humidified chamber. Colour was developed with 3,3'-diaminobenzidine (DAB) and H_2O_2, and slides were counterstained with methyl green. For NF-κB staining, heavy metal intensification was carried out by nickel-cobalt DAB.

DETECTION OF DNA FRAGMENTATION BY TERMINAL DEOXY (D)-UTP NICK END LABELLING (TUNEL) ASSAY

For analysis of DNA fragmentation in histological sections, a TUNEL assay was performed according to the method of Gavrieli *et al*.[19] Briefly, tissue sections were digested with 1 mg/ml proteinase K (Wako, Osaka, Japan) for 7.5 minutes at 37°C, and the slides were then washed with PBS three times (five minutes each). Sections were rinsed once with distilled deionised water and incubated with TdT buffer (0.2 M Tris-HCl pH 6.6, 40 mM potassium cacodylate, and 5 mM cobalt chloride) alone. Then, TdT buffer containing 0.3 U/ml TdT (Boehringer Mannheim, Mannheim, Germany) and 10 mM biotinylated 16-dUTP (Boehringer Mannheim) was added to the sections, and the slides were incubated in a humidified atmosphere at 37°C for 60 minutes. The reaction was terminated by 50 mM Tris-HCl pH 7.5, and washing four times for 10 minutes with PBS. Endogenous peroxidase was inactivated by immersing the sections in 0.3% H_2O_2 in methanol for 15 minutes at room temperature, followed by washing in PBS. After incubation with PBS containing 5% bovine serum albumin (BSA) and 500 μg/ml of goat IgG (Sigma, St Louis, MO) at room temperature for one hour to block non-specific binding, sections were incubated with horseradish peroxidase (HRP) labelled goat anti-biotin (Funakoshi, Japan) (1:100) diluted with 5% BSA in PBS at room temperature for two hours. After washing four times, they were visualised by incubation with H_2O_2 and DAB for 10 minutes. Control sections were obtained by staining in the absence of biotinylated 16-dUTP. We evaluated the TUNEL+ cells when intense staining with nuclear consolidation was seen. Cells with faint nuclear staining for TUNEL+ cells were not evaluated. We also observed 300 cells in the specimen for TUNEL assay within five layers from the lining cell layer.

INDUCTION OF APOPTOSIS IN CULTURED SYNOVIAL CELLS BY NF-κB INHIBITOR

Apoptosis of cultured synovial cells was induced by adding Z-Leu-Leu-Leu-aldehyde (LLL-CHO, Peptide Institute, Inc, Osaka), a potent NF-κB inhibitor. LLL-CHO is reported to protect IκB from degradation by proteasome and then inhibit dissociation of NF-κB from IκB. Thus LLL-CHO blocks nuclear translocation of NF-κB.[20] The synovial cells obtained after five passages were used in in vitro experiments and identified as fibroblast-like cells, as we previously reported.[17 21] Isolated synovial cells were cultured with or without 10 μM LLL-CHO for six hours in RPMI 1640 supplemented with 10% FBS, and were further incubated in the presence or absence of TNFα (200 U/ml), IL1β (20 U/ml), or monoclonal anti-Fas IgM antibody (1 μg/ml) for 18 hours. After cultivation for 24 hours, apoptosis of cultured synovial cells was quantified by the percentage of cells with hypodiploid DNA. Briefly, cells were fixed with 70% ethanol and treated with RNAase (100 μg/ml, Sigma), and then stained with propidium iodide (100 μg/ml, Sigma) for 30 minutes on ice. The stained cells were analysed using a flow cytometer (Epics XL, Beckman Coulter, Hialeah, FL) to detect the presence of hypodiploid DNA+ cells.

Intracellular caspase-3 activity in treated synovial cells was measured by PhiPhiLux G1D2 kit (MBL, Nagoya, Japan). Treated synovial cells were incubated with Asp-Glu-Val-Asp (DEVD) substrate containing rhodamine for 60 minutes in a CO_2 incubator at 37°C. After incubation, samples were analysed by flow cytometry to determine the percentage of intracellular active caspase-3+ cells. In some experiments, we added 200 μM Ac-DEVD aldehyde (DEVD-CHO, Peptide Institute, Inc, Osaka, Japan) in synovial cell culture, and followed this by examination of hypodiploid DNA+ cells to determine the functional significance of caspase-3.

DETERMINATION OF NUCLEAR NF-κB ACTIVITY BY ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

To determine DNA binding activity of nuclear NF-κB in cultured synovial cells, EMSA was performed by the use of a gel shift assay system (Promega Co, Madison, WI). Synovial cells were cultured with or without 10 μM LLL-CHO for six hours, and then further incubated in the presence or absence of TNFα, IL1β, or anti-Fas IgM for the time indicated. After cultivation, nuclear lysate was extracted and used for EMSA. In brief, binding reactions of 20 μl total volume contained 7.5 μg of nuclear proteins isolated from cultured synovial cells which were mixed with ^{32}P-radiolabelled double stranded oligonucleotide containing NF-κB binding site (5'-AGTTGAGGGGAC TTTCCCAGGC-3'), and 0.25 mg/ml of poly (dI-dC) (Sigma) in 10 mM Tris (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, and 4% glycerol. Reaction mixtures were incubated for 30 minutes at room temperature and analysed by 5% polyacrylamide gel electrophoresis. We measured each nuclear NF-κB density with the software NIH Image (1.61).

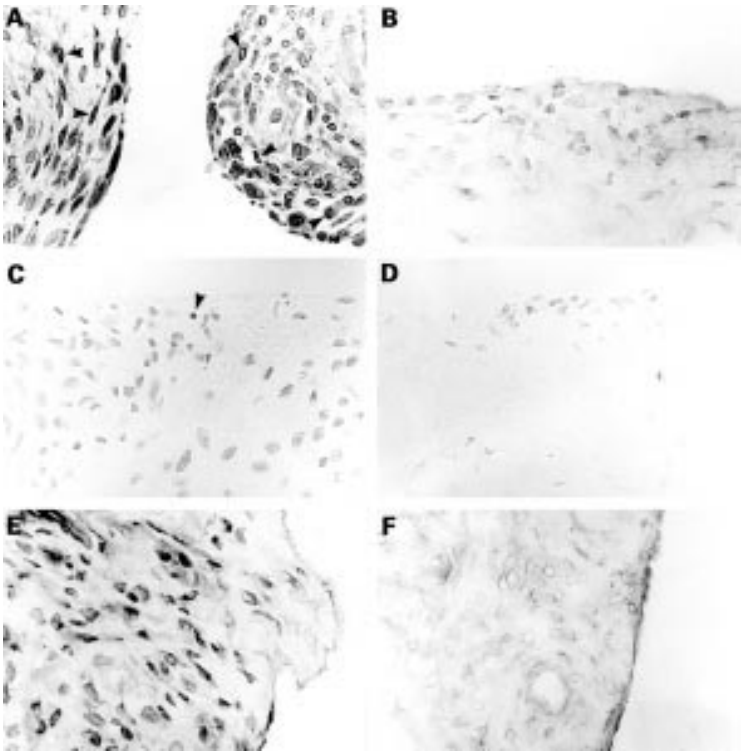

Figure 1 Expression of proliferating cell nuclear antigen (PCNA) (A, B), terminal deoxy (d)-UTP nick end labelling (TUNEL) assay (C, D), and NF-κB (E, F) in synovial tissues from patients with rheumatoid arthritis (RA) and osteoarthritis (OA). (A, B) PCNA staining in RA (A) and OA (B) synovial tissues. Note that the expression of PCNA was clearly detected in synovial cells of RA synovial tissue, but its expression was weak in OA synovial tissue. Arrowheads indicate representative PCNA+ cells. (C, D) TUNEL staining of RA (C) and OA (D) synovial tissues. A few synovial cells positive for TUNEL staining are present in RA synovial tissue, but those were rarely detected in OA synovial tissue. (E, F) NF-κB expression in RA (E) and OA (F) synovial tissue. Note the strong nuclear NF-κB expression in synovial cells of patients with RA. Nuclear NF-κB expression was not clear in synovial cells of patients with OA. Results shown are representative results from 10 patients with RA and 10 with OA. Note that no significant staining was observed by the use of control immunoglobulin (PCNA and NF-κB) or performed in the absence of biotinylated 16-dUTP (TUNEL) (data not shown). Magnification ×400.

DETECTION OF IAP PROTEINS BY WESTERN BLOTTING

Treated synovial cells were washed three times with PBS, and lysed by the addition of extraction buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 1% NP-40, and 100 μg/ml phenylmethylsulphonylfluoride). Protein concentrations in cell extracts were determined by the protein assay kit (Bio-Rad, Melville, NY). Identical amounts of protein for each lysate (20 μg/well) were subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to a polyvinylidene difluoride filter, which was subsequently blocked for one hour using 5% non-fat dried milk in Tris buffered saline (TBS) containing 0.5% Tween 20 (TBS-T). The filter was then washed with 1% non-fat dried milk in TBS-T, and incubated at room temperature for one hour in a 1:500 dilution of mouse antihuman XIAP mAb (MBL) or 1:1000 dilution of rabbit antihuman cIAP2 polyclonal antibody (Santa Cruz, CA). The filter was washed with TBS-T and incubated with 1:1000 dilution of sheep antimouse and antirabbit IgGs, coupled with HRP. The enhanced chemiluminescence system (Amersham, Buckinghamshire, UK) was used for detection. The density of expression of each protein was also measured with the software NIH Image (1.61).

STATISTICAL ANALYSIS

All data were expressed as means (SEM). Differences between groups were examined for statistical significance using Student's *t* test. A p value less than 0.05 indicated a significant difference.

Results

IMMUNOHISTOCHEMICAL ANALYSIS OF RA SYNOVIAL TISSUE

We initially examined the expression of PCNA, presence of apoptotic nuclei, and nuclear NF-κB in synovial cells. Figure 1A shows that the percentage of PCNA+ synovial cells was significantly higher in RA synovial tissue than that in synovial tissues of patients with OA (fig 1B) (98.8 (30.5)/300 cells *v* 11.8 (9.6)/300 cells, respectively, $p<0.01$, data from 10 subjects with RA and 10 with OA). TUNEL+ synovial cells were rarely detected in OA synovial tissues (fig 1D), but a few were seen in RA synovial tissues (fig 1C; the percentage of TUNEL+ synovial cells in RA synovial tissue

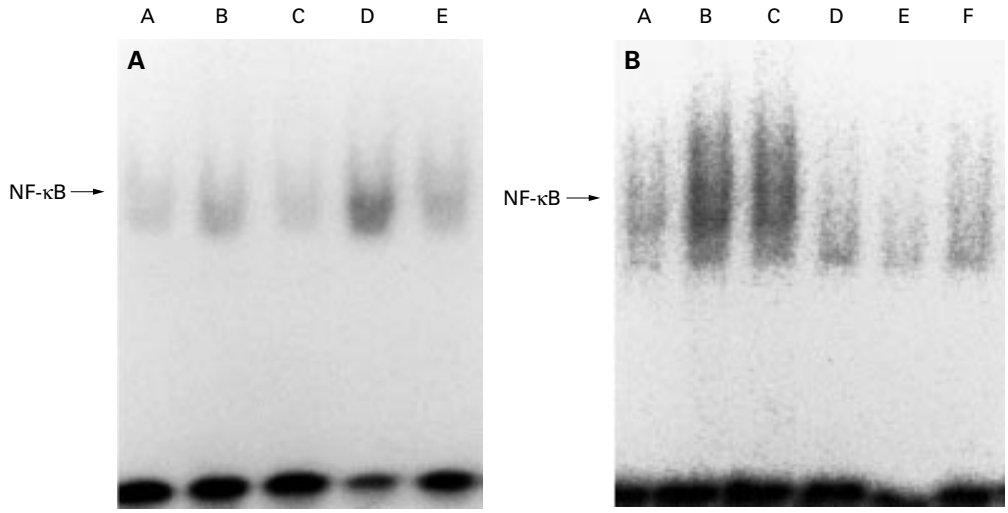

Figure 2 NF-κB nuclear translocation in cultured synovial cells determined by EMSA. (A) Note the presence of a weak basal NF-κB nuclear activity in unstimulated synovial cells, and markedly increased activity in cells stimulated by tumour necrosis factor α (TNFα) or interleukin 1β (IL1β). Synovial cells from patients with rheumatoid arthritis (RA) were cultured with or without TNFα (200 IU/ml) or IL1β (20 IU/ml). After cultivation, NF-κB nuclear translocation was examined by EMSA. A = unstimulated synovial cells; B = synovial cells treated with TNFα for one hour; C = synovial cells treated with TNFα for three hours; D = synovial cells treated with IL1β for one hour; E = synovial cells treated with IL1β for three hours. (B) Suppression of NF-κB nuclear translocation in synovial cells by Z-Leu-Leu-Leu-aldehyde (LLL-CHO). Synovial cells were initially cultured with 10 μM LLL-CHO for six hours, and further incubated with TNFα or IL1β for one hour. After incubation, NF-κB nuclear activity was examined by EMSA. Note that nuclear NF-κB activity in unstimulated, TNFα stimulated, or IL1β stimulated synovial cells was almost suppressed by LLL-CHO. A = unstimulated synovial cells; B = synovial cells stimulated with TNFα ; C = synovial cells stimulated with IL1β ; D = unstimulated synovial cells treated with LLL-CHO; E = TNFα stimulated synovial cells in the presence of LLL-CHO; F = IL1β stimulated synovial cells in the presence of LLL-CHO. Results shown are representative data of five experiments; similar results were obtained from four other experiments.

was <1%). These results suggest that proliferation of synovial cells exceeds apoptotic cell death in RA synovial tissue, thus favouring hyperplasia of synovial tissue. We next examined the expression of nuclear NF-κB in synovial tissues. Figure 1E shows that strong NF-κB nuclear expression in synovial cells was detected in RA synovial tissues compared with OA synovial tissues (fig 1F). PCNA and nuclear NF-κB expression were seen in synovial cells located in both synovial lining and sublining layers (figs 1A and E). We carried out an immunohistochemical analysis of 10 patients with RA and 10 with OA, and representative results are shown in fig 1. Similar results were obtained from the other nine experiments.

REGULATION OF SYNOVIAL CELL APOPTOSIS IN VITRO BY INHIBITION OF NF-κB NUCLEAR TRANSLOCATION

In the next step, we examined whether NF-κB exhibits anti-apoptotic activity in cultured synovial cells. Figure 2A shows that small basal nuclear NF-κB activity was determined in cultured RA synovial cells. Basal nuclear NF-κB activity was rarely detected in cultured OA synovial cells (data not shown). When RA synovial cells were incubated with TNFα or IL1β, nuclear NF-κB activity was markedly augmented in these cells (fig 2A). Treatment of synovial cells with anti-Fas mAb did not alter NF-κB nuclear activity (data not shown). The use of LLL-CHO clearly suppressed nuclear NF-κB activity in unstimulated, TNFα stimulated, or IL1β stimulated synovial cells (fig 2B). The density of nuclear NF-κB activity in EMSA was quantified by densitometric analysis as described in "Patients and methods"; three studies gave almost the same results. Cultured synovial cells in vitro did not undergo spontaneous apoptosis. However, treatment of the cells with LLL-CHO induced apoptotic cell death with an increase of caspase-3 activity, though the amount of increase was not so obvious (fig 3). TNFα or IL1β alone did not induce apoptosis of synovial cells (fig 3). When TNFα stimulated or IL1β stimulated synovial cells were used as target cells, the effect of LLL-CHO on the induction of apoptosis and caspase-3 activation was enhanced, compared with unstimulated synovial cells (fig 3). As previously described, Fas mediated apoptosis of synovial cells with an increase of caspase-3 activity was seen (fig 4). Furthermore, the Fas effect was clearly increased when LLL-CHO treated synovial cells were used; nuclear NF-κB activity in these cells was already suppressed (fig 4). The addition of DEVD-CHO almost suppressed apoptotic cell death of synovial cells induced by LLL-CHO or anti-Fas mAb (data not shown).

EXPRESSION OF IAP FAMILY PROTEINS IN SYNOVIAL CELLS DURING LLL-CHO INDUCED APOPTOSIS

The family of inhibitors of apoptosis proteins (IAPs) plays an important part in protecting cells from apoptotic stimuli, and their expression is regulated by NF-κB in certain cells.[22 23] We examined the expression of cIAP2 and XIAP in synovial cells cultured in the presence

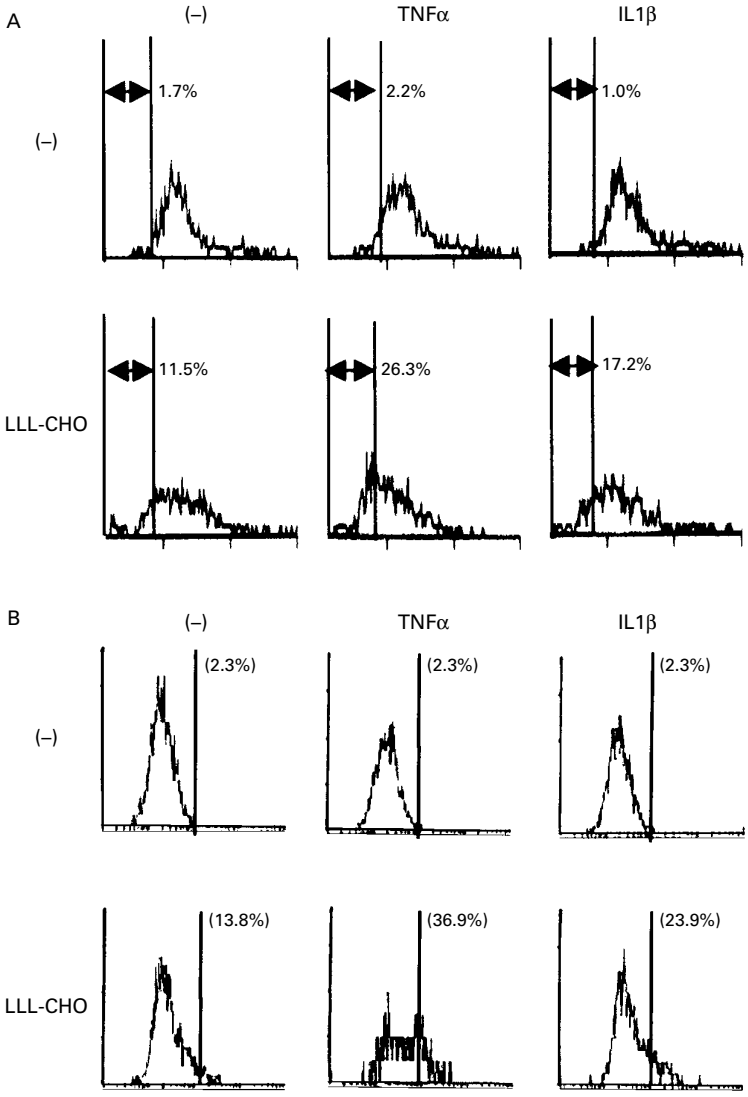

Figure 3 Induction of apoptosis and caspase-3 activation in synovial cells by Z-Leu-Leu-Leu-aldehyde (LLL-CHO). Synovial cells from patients with rheumatoid arthritis (RA) were initially cultured with or without LLL-CHO (10 μM) for six hours, and further incubated in the presence or absence of tumour necrosis factor α (TNFα; 200 IU/ml) or interleukin 1β (IL1β; 20 IU/ml) for an additional 18 hours. After incubation, apoptotic cell death (A) and activation of caspase-3 (B) were examined as described in the text. Note that neither apoptosis nor caspase-3 activation was obvious in unstimulated, TNFα stimulated, or IL1β stimulated synovial cells. Treatment of synovial cells with LLL-CHO induced apoptosis with an increase of intracellular caspase-3 activity, which was higher in TNFα or IL1β stimulated synovial cells. Results are representative data of six experiments; similar results were obtained from five other experiments.

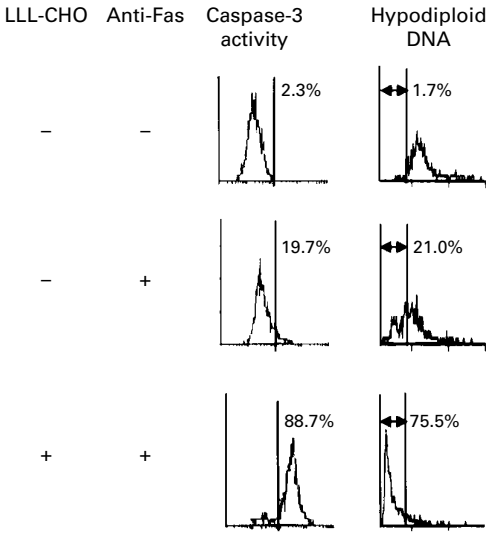

Figure 4 Treatment of synovial cells with Z-Leu-Leu-Leu-aldehyde (LLL-CHO) augments Fas mediated apoptosis of synovial cells. Synovial cells from patients with rheumatoid arthritis (RA) were initially cultured with or without LLL-CHO (10 μM) for six hours, and further incubated in the presence or absence of anti-Fas monoclonal antibody (mAb; 1 μg/ml) for an additional 18 hours. After incubation, apoptotic cell death and activation of caspase-3 were examined as described in the text. LLL-CHO enhanced anti-Fas mAb induced apoptosis with an increase of caspase-3 activity. Results are representative data of six experiments; similar results were obtained from five other experiments.

or absence of TNFα, IL1β, or anti-Fas mAb, with or without LLL-CHO. Figure 5 shows that both cIAP2 and XIAP were expressed in unstimulated synovial cells. Their expression was suppressed in TNFα stimulated synovial cells, but unchanged in IL1β stimulated synovial cells (fig 5). Expression of cIAP2 and XIAP was not affected by LLL-CHO in unstimulated or TNFα stimulated synovial cells. However, treatment with LLL-CHO suppressed the expression of cIAP2 and XIAP in IL1β stimulated synovial cells (fig 5). Treatment of synovial cells with anti-Fas mAb inhibited the expression of cIAP2 and XIAP, particularly in the presence of LLL-CHO (fig 5). The intensity of each band in western blotting was confirmed by densitometric analysis and six experiments showed similar results.

Discussion

Our study showed that the number of proliferating synovial cells, determined by PCNA staining, was significantly higher than that of apoptotic synovial cells in synovial tissue of patients with RA, confirming that a net proliferation of synovial cells is positively regulated in RA synovium. Despite the relative suppression of apoptosis of synovial cells, high levels of several factors known to induce apoptotic cell death, such as TNFα and FasL+ T cells, are present in RA synovium.[6 24] These data suggest the presence of certain anti-apoptotic mechanisms in RA synovium. TNFα and IL1β, abundantly expressed in RA synovium,[5 6] are reported to activate NF-κB,[7 8] and this study showed a strong nuclear NF-κB expression in synovial cells. Thus activated NF-κB in RA synovial cells might act against apoptosis. Alternatively, it is possible that inhibition of NF-κB augments apoptotic cell death of synovial cells stimulated by TNFα and IL1β.

Small basal nuclear NF-κB activity was determined in cultured synovial cells isolated from RA synovial tissue, and its activity was markedly increased by treatment of these cells with TNFα or IL1β. Suppression of basal nuclear NF-κB activity in cultured RA synovial cells by LLL-CHO resulted in increased caspase-3 activity and apoptosis of synovial cells, though the degree of those was not so obvious. Treatment of synovial cells with TNFα and IL1β alone did not induce apoptosis of cultured synovial cells. However, NF-κB suppression augmented apoptosis and caspase-3 activity in TNFα and IL1β stimulated synovial cells when compared with unstimulated synovial cells. Because apoptosis

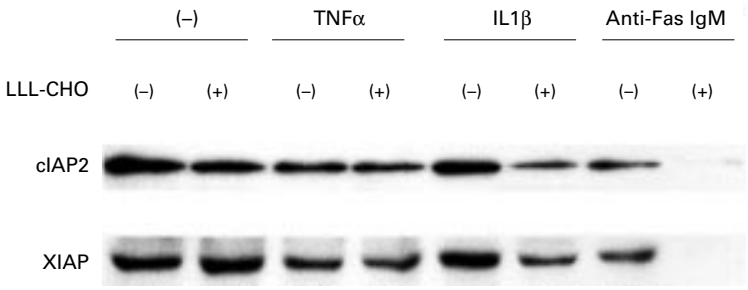

Figure 5 cIAP2 and XIAP expression in synovial cells determined by western blot analysis. Synovial cells from patients with rheumatoid arthritis (RA) were initially cultured with or without Z-Leu-Leu-Leu-aldehyde (LLL-CHO; 10 μM) for six hours, and further incubated in the presence or absence of tumour necrosis factor α (TNFα; 200 IU/ml), interleukin 1β (IL1β; 20 IU/ml), or anti-Fas mAb (1 μg/ml) for an additional 18 hours. After incubation, the expression of cIAP2 and XIAP in the cells was examined by western blot analysis as described in the text. Both cIAP2 and XIAP were expressed in unstimulated synovial cells, and the expression was down regulated by TNFα, but not by IL1β. Treatment with LLL-CHO did not affect the expression of cIAP2 and XIAP in unstimulated synovial cells or TNFα stimulated synovial cells. Expression of cIAP2 and XIAP in IL1β stimulated synovial cells was suppressed by LLL-CHO. Treatment of synovial cells with anti-Fas mAb inhibited the expression of cIAP2 and XIAP, which was significantly suppressed by LLL-CHO. Results are representative data of six experiments; similar results were obtained from five other experiments.

of synovial cells induced by LLL-CHO was almost suppressed by DEVD substrate, the major effector molecule inducing apoptosis of cultured synovial cells in this study seems to be caspase-3. The effect of NF-κB inhibition in synovial cells was more prominent in the presence of TNFα and IL1β, suggesting that NF-κB inhibition effectively triggers apoptosis of synovial cells in the inflamed synovium such as RA, and emphasising the importance of NF-κB as a therapeutic target molecule for RA. The protective function of NF-κB in TNFα induced apoptosis has already been recognised.[9–11 23] Furthermore, our study showed a possible link between NF-κB and caspase cascade in IL1 receptor signalling. Our study found that Fas mediated apoptosis and caspase-3 activation in synovial cells was also clearly augmented in cells with inhibited NF-κB activity. These data indicated, in addition to TNFα and IL1β induced cytotoxicity, that NF-κB inhibition may potentiate Fas mediated apoptosis of synovial cells in RA synovial tissue, leading to remission of synovitis in patients with RA.

NF-κB regulates the expression of molecules that act against apoptosis. cIAP2 and XIAP suppress the activation of caspase-3, and their expression is positively regulated in human endothelial cells by TNFα through the induction of NF-κB nuclear translocation.[22 25–27] However, the expression of cIAP2 and XIAP in synovial cells was clearly down regulated by TNFα. NF-κB nuclear activity was suppressed in unstimulated or TNFα stimulated synovial cells by LLL-CHO, but the expression of cIAP2 and XIAP was not affected in these cells, indicating that these proteins do not act to suppress the activation of caspase-3 in unstimulated or TNFα stimulated synovial cells. The expression of cIAP2 and XIAP in synovial cells was not changed by IL1β, but the expression was clearly inhibited by LLL-CHO in IL1β stimulated synovial cells, suggesting that activated NF-κB in IL1β stimulated synovial cells may be important for maintaining the expression of cIAP2 and XIAP to suppress the activation of caspase-3. Thus our data suggest that the expression of IAPs by NF-κB is regulated in a cell type-specific manner, and other NF-κB regulated molecules, such as TNF receptor associated factor 1 (TRAF1) and TRAF2 by TNFα or TRAF6 by IL1β,[28] may be involved in regulating the activation of caspase-3 in human synovial cells. Akt may be a target molecule to induce the activation of NF-κB in the present study because Akt is activated by TNFα and IL1β, stimulating nuclear translocation of NF-κB, and shows an anti-apoptotic effect.[29–31]

Although signalling through Fas did not alter NF-κB nuclear translocation in synovial cells, the activation of caspase-3 was noted in anti-Fas mAb treated synovial cells. Inhibition of NF-κB nuclear translocation markedly augmented Fas mediated synovial cell apoptosis, in which the activation of caspase-3 was also clearly increased. The expression of cIAP2 and XIAP in anti-Fas mAb treated synovial cells was suppressed, and in addition, it was further reduced in LLL-CHO treated synovial cells with anti-Fas mAb. Because IAPs can be a substrate for caspase-3,[32] activated caspase-3 may degrade cIAP2 and XIAP of anti-Fas mAb treated synovial cells. Otherwise, signals delivered through Fas in synovial cells may suppress the expression of cIAP2 and XIAP, and inhibition of NF-κB activity may cooperate with Fas elicited signalling by inhibiting expression in cIAP2 and XIAP, thus accelerating caspase-3 activation. Further studies are necessary to clarify the molecular mechanisms that activate caspase-3 after inactivation of NF-κB in Fas mediated apoptosis of synovial cells.

This study showed that NF-κB inhibition effectively triggered apoptosis of synovial cells stimulated by TNFα, IL1β, and anti-Fas mAb. Previous studies using animal models of arthritis have shown the beneficial therapeutic effects of interfering with the signalling through TNFα or IL1 receptors.[33] Furthermore, our study showed that the use of an NF-κB inhibitor preferentially eliminated TNFα or IL1β stimulated synovial cells through apoptosis even after signals were transduced through their receptors. The clinical usefulness of Fas mediated apoptosis in synovial cells for the treatment of RA has been shown in animal studies,[34] and our results showed that its effect can also be augmented by the simultaneous use of NF-κB inhibitor. NF-κB inhibition may be a potentially important therapeutic approach for patients with RA.

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