Reduction of Tumor Necrosis Factor Induced Nuclear Factor-κB Nuclear Translocation and DNA Binding by Dexamethasone in Human Osteoarthritic Synovial Tissue Explants

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ABSTRACT. *Objective.* The antiinflammatory effects of glucocorticoids are mediated by several mechanisms, including inhibition of nuclear factor-κB (NF-κB) nuclear translocation and DNA binding. This mechanism is not evident in some cell types, including endothelial cells and rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS). We determined the effect of glucocorticoids and tumor necrosis factor (TNF) on nuclear localization and DNA binding of the transcription factor NF-κB in osteoarthritic (OA) synovial tissue.

Methods. Explants of synovial tissue from patients undergoing joint replacement surgery for arthritis were placed in culture and treated with dexamethasone 10^{-6} M for 18 h and again at 30 min prior to stimulation with TNF for a further 30 min. NF-κB and AP-1 DNA binding activities were determined by electrophoretic mobility shift analysis of nuclear extracts prepared from 6 whole tissue explants. Nuclear localization of NF-κB was determined by quantitative immunohistochemistry for Rel-A(p65) in thin sections of 5 synovial tissue explants.

Results. TNF induced NF-κB nuclear translocation and DNA binding in all OA synovial tissue explants, although there were no consistent effects on AP-1 DNA binding. Dexamethasone reduced TNF stimulated nuclear translocation of RelA(p65) in all 5 OA synovial explants analyzed by immunohistochemistry. Dexamethasone partially decreased NF-κB DNA binding in 5 of 6 TNF stimulated explants and 4 of 6 unstimulated explants. In cultured rheumatoid arthritis and OA fibroblast-like synoviocytes and Mono Mac 6 cells the effects of dexamethasone on NF-κB DNA binding were not evident.

Conclusion. Dexamethasone partially inhibits TNF induced NF-κB DNA binding in human synovial tissue. It is feasible to use explants of intact fresh human synovium as a substrate for the action of antirheumatic drugs targeting a transcription factor. (J Rheumatol 2002;29:787–95)

Key Indexing Terms:
NUCLEAR FACTOR–κB
GLUCOCORTICOID
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TUMOR NECROSIS FACTOR

Several observations indicate that the transcription factor nuclear factor-κB (NF-κB) is likely to play a crucial role in the pathology of synovial inflammation in humans and rodents. NF-κB is a heterodimer of 2 proteins, typically RelA(p65) and p50 (NF-KB1), although other family members such as Rel, RelB, v-Rel, and p52 (NFKB2) may take part[1]. Transcriptional activity of NF-κB is largely controlled by its sequestration in the cytoplasm, where it is bound to an inhibitor, IκBα or IκBß. After stimulation of the cell, IκB are phosphorylated and degraded by proteasomes, releasing NF-κB for translocation to the nucleus, where it binds the promoter sequences of numerous proinflammatory genes. Localization of RelA(p65) and p50 in the nuclei of synovial cells in rheumatoid arthritis (RA) and osteoarthritis (OA) is evidence that this signal transduction pathway is activated in human disease[2,3]. Additional requirements for transcriptional activity of NF-κB are the assembly and phosphorylation of proteins in the transcription complex[4-7].

NF-κB is particularly important in the regulation of gene expression in inflammation, typically as a heterodimer of RelA(p65) and p50. Inducers of NF-κB include tumor necrosis factor (TNF), interleukin 1ß (IL-1ß), platelet derived growth factor, oxidative stress, viral products, and bacterial cell wall products such as lipopolysaccharide

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(LPS). In turn NF-κB can activate the transcription of cytokines (TNF, IL-1ß, IL-6, IL-8), adhesion molecules (intercellular adhesion molecule-1, vascular cellular adhesion molecule-1, E-selectin), and enzymes [inducible nitric oxide synthase, cyclooxygenase-2 (COX-2), cPLA$_2$] that form the main known contributors to the inflammatory process. These inducers and targets of NF-κB closely resemble the list of inflammatory mediators in human arthritis, suggesting a positive feedback mechanism with NF-κB in a central position in the amplification and possibly perpetuation of synovitis. Indeed, inhibition of NF-κB by overexpression of IκBα in rheumatoid synovium *ex vivo* leads to inhibition of inflammatory and destructive mediators[8]. A critical role for NF-κB is supported by the use of NF-κB decoys and IκB repressors that have effectively reduced inflammation and joint damage in a variety of rodent models of arthritis[9-11].

Inhibition of NF-κB activity is an important means by which glucocorticoids exert their antiinflammatory effects[1,12]. This takes place by several mechanisms. Ligand activated glucocorticoid receptor (GR) can increase IκBα expression, thereby retaining NF-κB in the cytoplasm[13,14]. The formation of heterodimers between GR and RelA(p65) may prevent RelA(p65) from taking part in transcriptionally active NF-κB dimers, in addition to preventing the formation of GR homodimers[15,16]. Competition for limiting amounts of mutually important transcriptional coactivators such as p300 or CBP[4] may be another mechanism of mutual antagonism between GR and NF-κB. Glucocorticoids can also repress NF-κB mediated transcription by interfering with phosphorylation of RNA polymerase II without affecting DNA binding by NF-κB[6]. The mechanisms by which glucocorticoids act vary between cell types. For example, glucocorticoid mediated induction of IκBα expression and inhibition of NF-κB DNA binding[13,14] does not occur in RA fibroblast-like synoviocytes (FLS)[17] or endothelial cells[18].

Inhibition of NF-κB is a promising approach to the treatment of inflammation. Although this can often be achieved with glucocorticoids, the response is variable and adverse effects limit the usefulness of this important class of drug. We examined the effect of dexamethasone in synovial explants to determine the feasibility of using fresh human tissue as a substrate for pharmacological studies and to measure the magnitude of inhibition of NF-κB DNA binding in this tissue.

MATERIALS AND METHODS

Synovial tissue preparation. Synovial tissue specimens for explant culture were obtained from patients undergoing joint replacement surgery for OA. Samples for explant culture were immediately exposed to RPMI 1640 medium containing 2% fetal bovine serum (FBS). The synovial lining surface of the tissue was identified and 5 strips about 2 × 8 mm were excised to a depth of roughly 2 mm. Each strip was divided into 4 fragments of roughly 2 mm^3 and placed into 4 different wells of a 6 well tissue culture plate. The 4 wells, each containing 5 representative fragments, were cultured with different treatments: vehicle control, TNF 10 ng/ml (R&D Systems), dexamethasone 10^{-6} M, and TNF plus dexamethasone. For immunohistochemistry all 5 fragments from a single treatment were snap frozen into one block of OCT compound and stored at –80°C. Specimens for transcription factor DNA binding analysis were minced prior to preparation of nuclear extracts.

Cell culture. Human FLS were established from tissue obtained from arthritis patients at the time of joint surgery, essentially as described[19]. Tissue was minced and enzymatically dissociated by treatment with trypsin (0.5%)/EDTA (5.3 mM) for 15 min followed by collagenase (200 U/ml) for 15 min at 37°C. The pelleted tissue was passaged through 19 gauge needles twice, and resulting cells were cultured in 25 cm^2 flasks in Dulbecco's modified Eagle's (DMEM)/Ham's F12 medium, 10% FBS, penicillin, streptomycin, and amphotericin. Cells were cultured in antibiotics for the first passage only. The adherent cell population, termed FLS, was used between passage 5 and 9. For experiments, cultures in 150 cm^2 flasks were washed twice in phosphate buffered saline (PBS) and then received fresh DMEM/Ham's F12 plus 0.1% bovine serum albumin (BSA) (fatty acid-free and low endotoxin; Boehringer Mannheim) before cytokine addition. Mono Mac 6 cells were obtained from Prof. H.W.L. Ziegler-Heitbrock, Institut fuer Immunologie, Universitaet Muenchen, Muenchen, Germany.

Nuclear extract preparation. A nuclear protein extraction procedure was used with modifications[20]. Pieces of synovial tissue (1 mm^3) were washed twice by centrifugation in PBS, and resuspended at 4°C in 400 µl Buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl$_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 µg/ml aprotinin, 100 µg/ml leupeptin). Tissues were allowed to swell on ice for 15 min and then were homogenized in a teflon-glass Potter-Elvehjem homogenizer, 40 µl of 10% Nonidet P40 (Fluka) was added, and the tube was vigorously vortexed for 45 s. After centrifugation at 4°C for 1 min at 14,000 g the supernatant was removed, the pellet was washed twice in 100 µl Buffer A and pelleted at 14,000 g for 1 min. It was then exposed to 40 µl of high salt Buffer C (10 mM Hepes, pH 7.9, 420 mM KCl, 1.5 mM MgCl$_2$, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 100 µg/ml aprotinin, 100 µg/ml leupeptin). The tube was vortexed and rocked vigorously on a shaking platform for 15 min at 4°C and centrifuged at 14,000 g for 10 min. Nuclear extracts were aliquoted and frozen at –80°C. The protein concentration of extracts was typically 2–6 µg/µl, as determined by a dye binding method (Biorad, Sydney, Australia). Buffer C was used to normalize variations in the protein concentration of samples to be compared within the same experiment. Nuclear extracts were also prepared from cultured Mono Mac 6 cells and FLS.

Electrophoretic mobility shift assay. EMSA was performed as follows: binding reactions, containing 3–8 µg of nuclear protein, poly(dI-dC)·poly(dI-dC) (Pharmacia, Sydney, Australia) at a concentration of 0.25 µg per µg total protein, 1–2 ng ^{32}P labeled double stranded oligonucleotide probe and DNA binding buffer to 20 µl [20 mM Hepes, pH 7.9 (cells) or 10 mM Tris, pH 7.5 (tissue), 1 mM EDTA, pH 8.0, 60 mM KCl, 5 mM MgCl$_2$ (AP-1 only), 1 mM DTT, and 12% glycerol] were mixed and incubated for 30 min at room temperature. The DNA binding complexes were separated by nondenaturing 5% polyacrylamide gel electrophoresis in 0.25× tris-borate-EDTA buffer, pH 8.3, at 150 V for 2–3 h. Dried gels were imaged by autoradiography and specific complexes quantified by capturing with a PhosphorImager (Molecular Dynamics 445 SI, Molecular Dynamics, Sunnyvale, CA, USA) and analyzed using IP Lab Gel H software (Signal Analytics, Vienna, VA, USA). Data were normalized by assigning a value of 1 to the lowest phosporimage quantity. The top strand sequences used for the NF-κB or AP-1 probes were as follows: NF-κB, 5′-AGTTGAGG GGACTTTCCCAGGC-3′; AP-1, 5′-CTAGTGATGAGTCAGCCGGATC-3′.

Western blots. Synovial tissue fragments were homogenized with a FastPrep FP120 cell disruptor (Bio 101, La Jolla, CA, USA) in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl$_2$, 1 mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF). Homogenates were centrifuged (13,000 g, 10

min, 4°C), supernatants collected, protein quantified by the DC assay (Bio-Rad), and equal amounts of protein (20 μg) were fractionated on 4–20% SDS-polyacrylamide gels (Novex, San Diego, CA, USA) and transferred to nitrocellulose membrane (Amersham, Cleveland, OH, USA). Membranes were blocked with 5% skim milk powder in Tris buffered saline (TBS)/Triton (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% v/v Triton X-100), incubated with primary antibodies in TBS/BSA (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5% w/v BSA) for 2 h at room temperature, washed, incubated with horseradish peroxidase (HRP) conjugated secondary reagent for 1 h at room temperature, and washed again. HRP was detected with enhanced chemiluminescence (Renaissance reagent, NEN Life Sciences) and visualized by autoradiography. COX-2 was detected with goat polyclonal primary antibody (sc1745, diluted 1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by Protein G-HRP (10-1223, diluted 1:10,000; Zymed). ß-Actin was detected with mouse mono-clonal antibody (A-5441, diluted 1:3000; Sigma, St. Louis, MO, USA) followed by anti-mouse Ig-HRP antibody (NA931, diluted 1:3000; Amersham).

Immunohistochemistry. Immunohistochemistry for NF-κB(p65) was performed as described[2]. Frozen synovial sections (6 μm) on Superfrost (Tissue-Tek) glass slides were thawed and all subsequent steps performed at room temperature. Sections were air dried for 1 h, fixed in chloro-form:acetone (1:1) for 10 min, air dried a further 1 h, rehydrated in 0.05 M Tris HCl, pH 7.4, blocked with 20% goat serum for 20 min, and incubated 30 min with polyclonal rabbit anti-NF-κB/p-65/(C-20) IgG (Santa Cruz Biotechnology) diluted 1:250 (0.4 μg/ml) in 2% goat serum. The sections were washed, incubated 30 min with peroxidase conjugated goat anti-rabbit IgG (human/mouse adsorbed; Southern Biotechnology) diluted 1:250 in 2% goat serum, washed again, and developed with DAB and H_2O_2. Adjacent sections were counterstained with hematoxylin.

Quantitation of immunohistochemistry. Image analysis was performed using a Leica DMR microscope with Leica Q500MC image processing and analysis system. Slides were arranged in random order and analyzed by 2 observers (CM and MLH), who were unaware of their order. For each tissue section, images of 10 randomly selected fields (×40) along the synovial lining were captured, so that each field contained synovial lining layer and the adjacent subintimal area. For each field the number of RelA(p65) stained nuclei were counted, the area of tissue analyzed was measured, and a value for the number of RelA(p65) positive nuclei per unit area derived. The same 10 fields were identified in adjacent hematoxylin stained sections and the total number of nuclei per unit area derived for each field. The total number of nuclei per field was typically in the range 100 to 400. The proportion of RelA(p65) positive nuclei was calculated as a ratio, (RelA positive nuclei per area)/(total nuclei per area), for each of the 10 fields, thus allowing calculation of mean ratio and standard error of the mean for each tissue section.

RESULTS

Characterization of NF-κB and AP-1 binding activities in OA synovium. Nuclear extracts from human OA synovial tissue (Figures 1, 2, and 3) were analyzed by EMSA, using double stranded ^{32}P labeled oligonucleotides representing the NF-κB and AP-1 DNA binding sites. Competition with as little as 10-fold unlabeled specific probe efficiently decreased the corresponding NF-κB and AP-1 complexes, whereas competition with 50-fold excess of unlabeled unre-lated probe had no effect, thus confirming sequence speci-ficity of DNA binding in human tissue (Figure 1). Addition of anti-p50 and anti-p65 antibodies to nuclear extracts resulted in supershifts that revealed the p50/p50 homo-dimers and p50/p65 heterodimers within the sequence-

specific NF-κB DNA binding complexes (Figure 2). Supershifts were not detectable with antibodies against RelB, cRel, p52, or jun. These experiments show that nuclear extracts prepared from fresh whole synovial tissue contain Rel-A(p65) and p50(NFKB1) that specifically bind the consensus NF-κB site when analyzed by EMSA.

The effect of dexamethasone and TNF in human synovial tissue explants. Explants of fresh human synovial tissue were treated with dexamethasone for 18 h and again at 30 min prior to stimulation with TNF. Nuclear extracts were prepared from the tissue 30 min after treatment with TNF or vehicle control. NF-κB DNA binding was induced by TNF treatment of synovial tissue from all 6 OA subjects (Table 1, Figures 3 and 6B). Dexamethasone decreased basal NF-κB DNA binding in tissue from 4 OA subjects, with minor changes in the other 2 (Subjects 5 and 6 in Table 1 and Figures 3 and 6B). Dexamethasone decreased TNF stimu-lated NF-κB DNA binding in tissue from 5 OA subjects with minor changes in the remaining subject, Subject 1. Inhibition of basal and TNF stimulated NF-κB DNA binding by dexamethasone has also been observed in a synovial explant from one RA subject[21]. Short and long exposures of autoradiographs were needed to visualize the effects of dexamethasone in TNF stimulated and unstimu-lated tissue, respectively. Only one exposure of each tissue is shown in Figure 3. Specific NF-κB DNA binding complexes were quantified by phosphorimaging (Table 1) and the Wilcoxon signed rank test was used to test for changes at the p < 0.05 level of significance. Reduction in NF-κB DNA binding in response to dexamethasone was statistically significant in TNF stimulated explants, whereas the effect of dexamethasone on the controls did not reach statistical significance. The effects of dexamethasone and TNF, either singly or combined, on AP-1 DNA binding revealed considerable variability (Figure 3). Variations between the 6 explants are attributed to the recognized interindividual variability in disease activity and response in the rheumatic diseases.

The biological consequences of dexamethasone and TNF in the synovial explant model were verified by Western blots for COX-2, which is known to be a NF-κB dependent gene product[22]. As expected, COX-2 expression was induced by TNF and inhibited by dexamethasone (Figure 4).

Quantitative immunohistochemistry of RelA(p65). Explants of OA synovial tissue from 5 subjects were treated twice with dexamethasone or control for 18 h and 30 min, stimu-lated with TNF or control for a further 30 min, and then snap frozen. Thin sections were stained for RelA(p65) and quan-tified by image analysis. The antibody used in these studies produces intense staining of nuclear RelA(p65) and only weak staining when RelA(p65) is located in the cytoplasm[2]. Development times for peroxidase staining were kept short in order to minimize cytoplasmic staining, thus enabling adjustment of the grey scale threshold on image analysis to

Table 1. Quantification of specific NF-κB DNA binding shown in Figure 3. Nuclear extracts from explants of OA synovium from 6 subjects treated *ex vivo* with dexamethasone and/or TNF analyzed by EMSA. Radioactive intensity was quantified by phosphorimaging of dried gels and normalized to a value of 1 unit for the lowest quantity. Reduction in NF-κB DNA binding in response to dexamethasone was statistically significant at the p < 0.05 level in TNF treated explants, according to the Wilcoxon signed rank test. These values are illustrated in Figure 6B.

	Subject					
	1	2	3	4	5	6
Control	4.73	10.18	7.55	8.55	3.86	8.36
Dexamethasone	1.00	5.82	3.64	1.73	3.77	8.86
TNF	15.32	17.05	20.55	16.55	13.05	12.68
TNF + dexamethasone	15.27	12.41	11.27	12.82	11.50	10.41
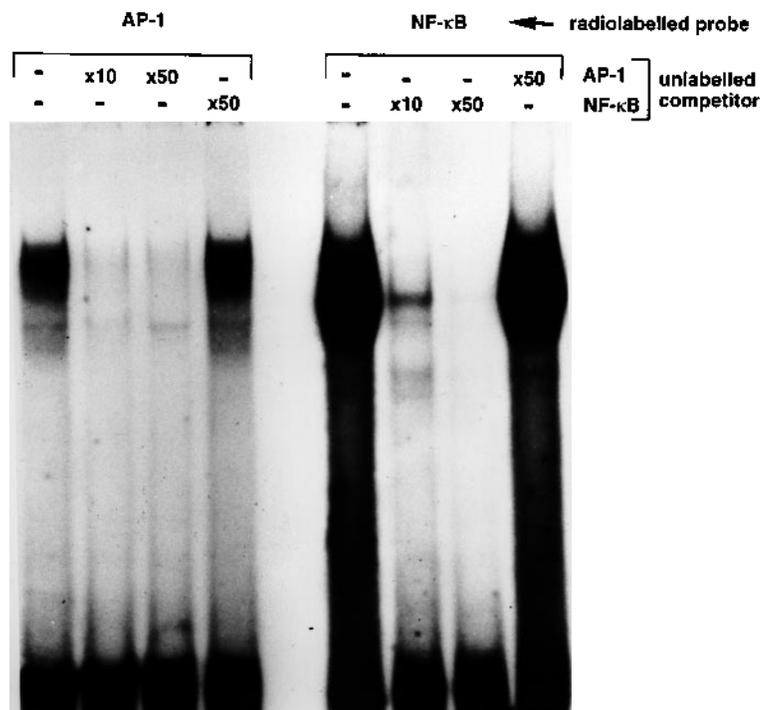
Figure 1. EMSA for AP-1 and NF-κB DNA binding in human OA synovial tissue. Nuclear extracts of tissue were incubated with radiolabeled double stranded DNA oligonucleotides representing the AP-1 and NF-κB consensus binding sites and analyzed by nondenaturing gel electrophoresis. Sequence-specific binding of the retarded complexes was shown by competition with 10 and 50-fold excess of unlabeled specific probe and 50-fold excess of unlabeled unrelated probes. Unbound probe has migrated to the bottom of the figure.

select only nuclear staining. Nuclei positive for RelA(p65) in each field were counted and expressed as a ratio of the total nuclei in the same region of a hematoxylin counterstained adjacent section (Figures 5 and 6A). As expected, unstimulated OA explants exhibited relatively low levels of immunostaining for nuclear RelA(p65), and only in the most intensely positive of the 5 specimens could a reduction in response to dexamethasone be observed (Figure 6A). TNF stimulation significantly increased nuclear RelA in all 5 explants (Figure 6A), presumably due to translocation of NF-κB to the nucleus. Prior treatment with dexamethasone

decreased TNF stimulated nuclear RelA(p65) in all 5 explants (Figure 6A), consistent with the effects in the DNA binding studies of explants (Figure 6B). The increase in mean values with TNF and the reduction with dexamethasone in TNF stimulated tissue were statistically significant at the p < 0.05 level according to the Wilcoxon signed rank test.

Effect of dexamethasone and TNF in cultured cells. Nuclear extracts of cultured FLS were analyzed by EMSA for NF-κB. Addition of anti-p50 and anti-p65 antibodies supershifted the p50/p50 homodimers and p50/p65 heterodimers

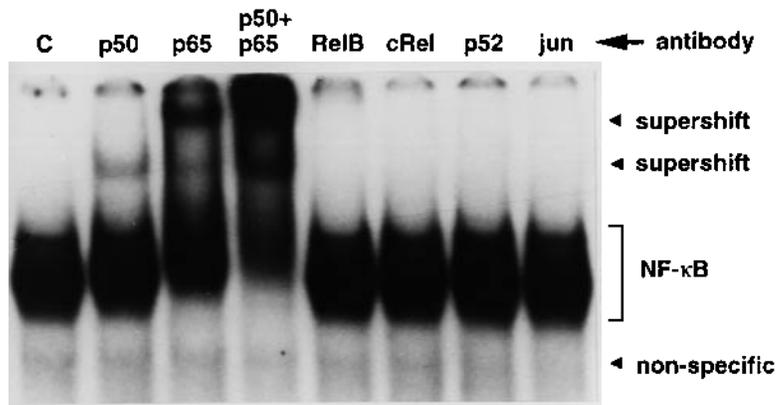*Figure 2*. EMSA for NF-κB DNA binding in human OA synovial tissue with supershifts to identify the presence of RelA(p65) and p50 in the sequence-specific complex. Nuclear extracts were incubated with antibodies against RelA(p65), p50, RelB, cRel, p52, and jun prior to addition of probe. The unbound probe is not shown.
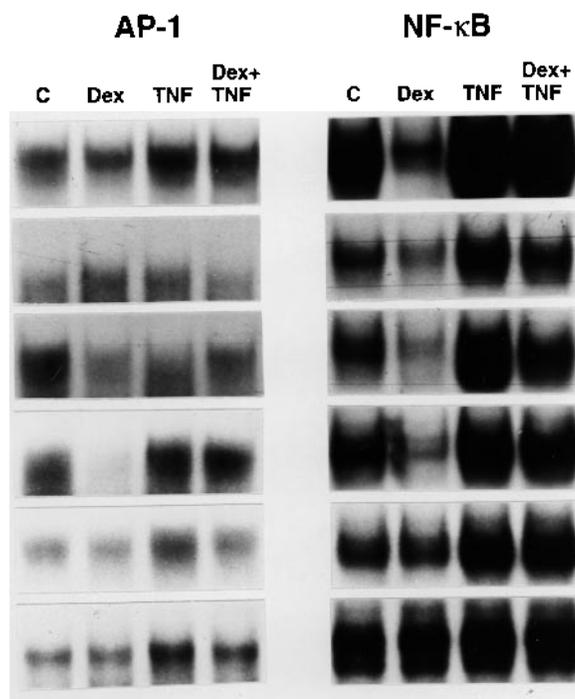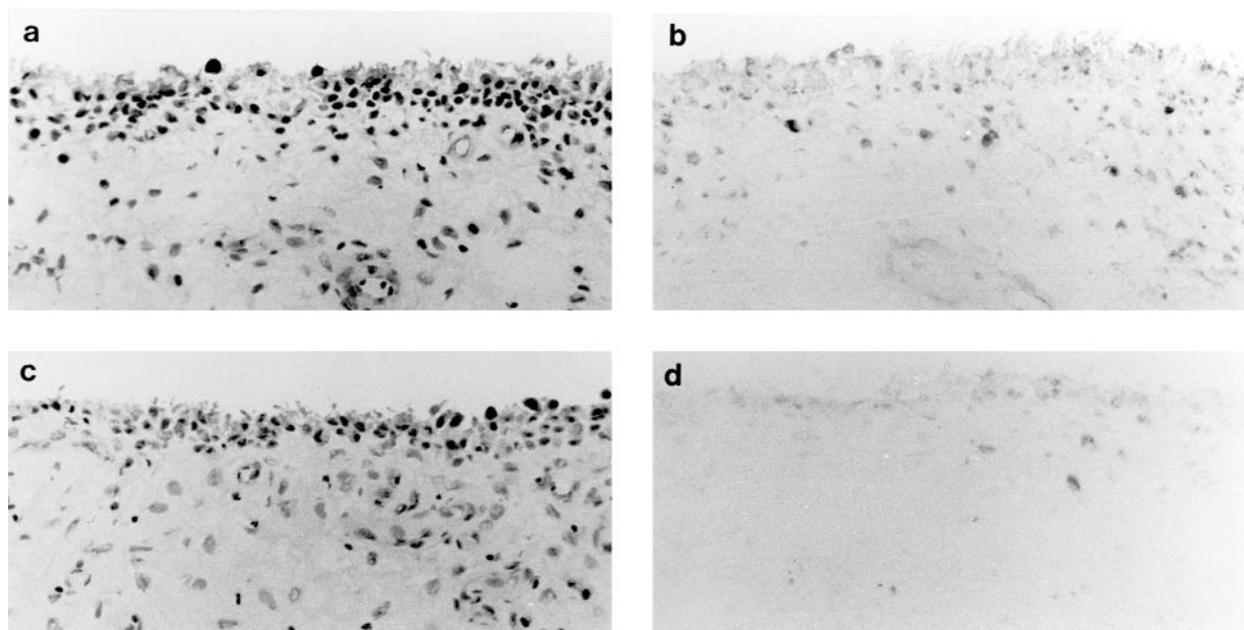

Figure 3. Effects of dexamethasone and TNF on AP-1 and NF-κB DNA binding in OA synovial tissue explants from 6 subjects. Explants dissected from synovial tissue removed at joint replacement surgery were placed in culture, treated twice with dexamethasone 10^{-6} M or control for 18 h and 30 min prior to stimulation with TNF 10 ng/ml for 30 min, harvested for extraction of nuclear proteins and analyzed by EMSA. The specific NF-κB DNA binding complexes were quantified by phosphorimaging of the dried gel (Figure 6B).

within the sequence-specific NF-κB DNA binding complexes, whereas RelB, cRel, and p52 were not detectable (Figure 7). A faster migrating complex that could be competed with 50-fold excess of unlabeled NF-κB probe was classified as nonspecific on the basis that it cannot be supershifted (Figure 7), is inefficiently competed with low concentration of unlabeled probe, and can be competed with poly(dI-dC)·poly(dI-dC)[23]. Cultured OA and RA FLS and Mono Mac 6 cells were pretreated with dexamethasone for

18 h and again at 30 min prior to stimulation with TNF for 30 min, and then harvested. Nuclear extracts were prepared and analyzed by EMSA (Figure 8). Controls included omission of TNF, dexamethasone, or both. All cell types exhibited weak basal NF-κB DNA binding that was strongly induced by TNF. Prior treatment with dexamethasone, with or without TNF, had no consistent effect on NF-κB DNA binding in multiple experiments with different OA and RA cultured FLS or with Mono Mac 6 cells. FLS exhibited
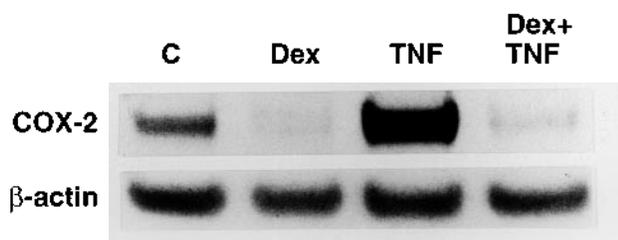
Figure 4. Western blot analysis of tissue explant lysates. Fresh OA synovial tissue explants were treated with dexamethasone and/or TNF as indicated, according to the same regimen as in Figure 3 except that tissue was harvested 18 h after stimulation with TNF to allow for protein accumulation. COX-2 and ß-actin control were detected by immunoblotting and chemiluminescence.

tive antirheumatic agents have been investigated using the EMSA for NF-κB DNA binding in nuclear extracts from FLS[17,30-32], macrophages[33,34], and joints of arthritic animals[35]. Our study confirms the feasibility of using explants of intact fresh human synovium as a substrate for the action of antirheumatic drugs targeting a transcription factor.

Glucocorticoids can inhibit NF-κB by several mechanisms, which vary according to the circumstances. For example, induction of IκBα by glucocorticosteroids occurs in some cell types[13,14], but not in endothelial cells[18] or RA FLS[17,36]. The presence of additional extracellular signals can alter the response, such as coadministration of FK506, which enhances dexamethasone induced nuclear transloc-

Figure 5. Photomicrographs of representative sections of TNF stimulated synovial tissue explants treated with and without glucocorticoids. Fresh explants were treated with dexamethasone 10^{-6} M (c and d) or control (a and b) for 18 h and 30 min prior to stimulation with TNF 10 ng/ml for 30 min. Immunoperoxidase staining for RelA(p65) in thin (6 μm) sections of snap frozen tissue was used to identify RelA(p65) positive nuclei (b and d), and hematoxylin counterstaining was used to identify all nuclei in the corresponding fields of adjacent thin sections (a and c). This figure shows a field from a TNF treated explant (a and b) and a field from a dexamethasone plus TNF treated explant (b and c) from one subject. Quantitative image analysis of 10 fields from each treatment arm of this particular subject revealed a ratio of 0.14 ± 0.02 RelA(p65) positive nuclei in the TNF treated explant and a ratio of 0.06 ± 0.01 RelA(p65) positive nuclei in the dexamethasone plus TNF treated explant (mean ± SEM). Data from all 5 subjects are represented in Figure 6A. Original magnification 200×.

strong constitutive AP-1 DNA binding that could not be further induced by TNF or inhibited by dexamethasone. Mono Mac 6 cells, which have some of the characteristics of synovial macrophages[23], exhibited negligible AP-1 DNA binding that was not induced by TNF.

DISCUSSION

The EMSA technique for NF-κB DNA binding has been used in many recent studies of arthritis. Biological substrates have included peripheral blood cells[24], cultured FLS[25-28], and whole human synovium[29]. Known and puta-

ation of GR with a corresponding decrease in DNA binding activity of NF-κB in RA FLS[37]. Similarly, it is possible that drug action is influenced by tissue matrix and diverse cell types in explants. These potential sources of variability highlight the value of using intact human tissue in pharmacological studies.

In this study dexamethasone treatment of fresh human OA synovial explants reduced TNF induced RelA(p65) nuclear translocation and NF-κB DNA binding, determined by immunohistochemistry and EMSA, respectively. In contrast, the effects of dexamethasone on NF-κB DNA
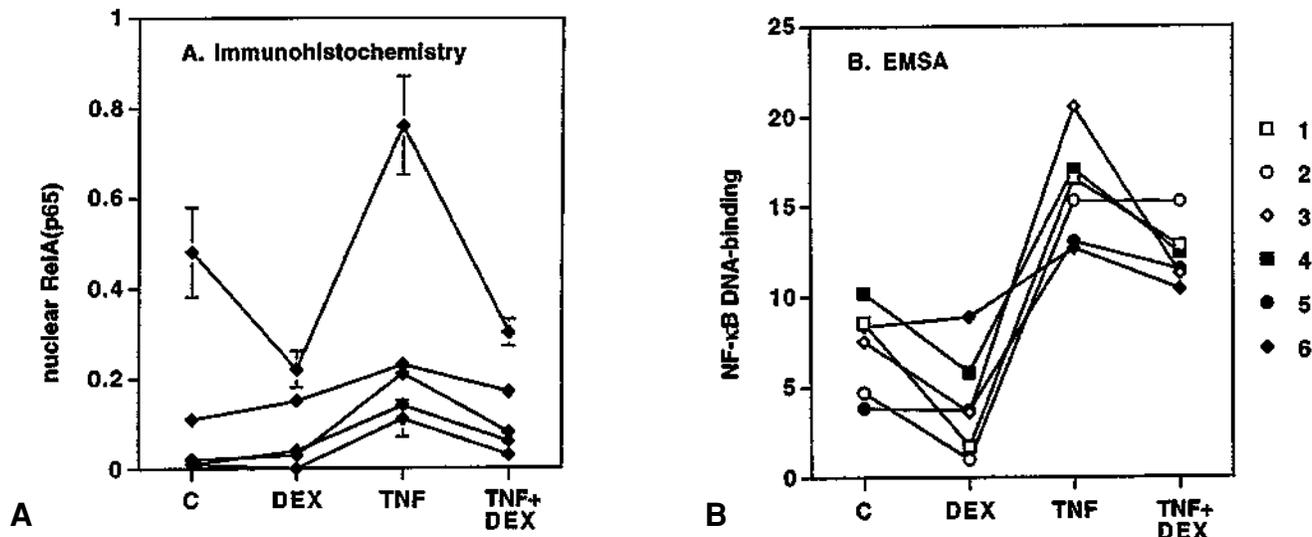
Figure 6. Effects of dexamethasone and TNF on RelA(p65) nuclear localization (A) and NF-κB DNA binding (B) in OA synovial tissue explants. Fresh synovial tissue explants were placed in culture and treated twice with dexamethasone 10^{-6} M or control for 18 h and 30 min prior to stimulation with TNF 10 ng/ml for 30 min. Immunohistochemistry for RelA(p65) was performed on thin sections of snap frozen explants from 5 subjects and expressed as the ratio of RelA(p65) positive nuclei to total nuclei. The mean ratio and SEM (error bars) for 10 fields within each section are shown (A). Relative values for the NF-κB DNA binding intensities in the EMSA of 6 subjects in Figure 3 and Table 1 were determined by phosphorimaging of the dried gels (B).
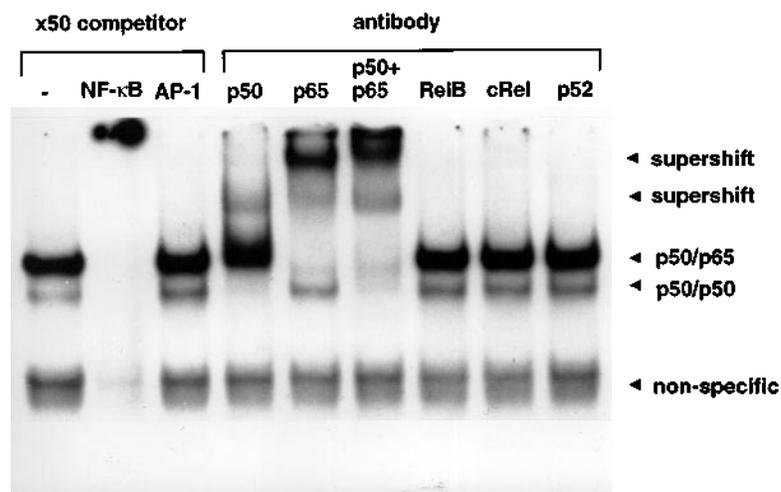

Figure 7. EMSA for NF-κB DNA binding in cultured OA FLS. Competitor unlabeled AP-1 and NF-κB probes have been added in 50-fold excess. Addition of antibodies against RelA(p65), p50, RelB, cRel, p52, and jun to the nuclear extracts resulted in supershifts identifying the presence of RelA(p65) and p50. The unbound probe is not shown.

binding in TNF stimulated cultured OA and RA FLS were not evident, consistent with a previous report for RA FLS[17]. Our observation that dexamethasone did not completely reverse TNF induced NF-κB DNA binding in human tissue could be explained by a mixed response within the tissue explants, with profound effects on some cell types and lack of effect in others. In this regard it remains to be seen whether the lack of glucocorticoid effect observed in cultured FLS[17] is also true of FLS within intact synovial explants, or if FLS are capable of response when in their normal matrix and cellular environment. Alternatively, incomplete inhibition of NF-κB DNA binding in human tissue may truly represent the limitations of glucocorticoid efficacy in most cell types. This phenomenon is illustrated in monocytic THP-1 cells, where LPS induced TNF expression is incompletely inhibited by dexamethasone, largely through incomplete inhibition of NF-κB and c-Jun/ATF-2 DNA binding[34]. In addition to the effects on nuclear translocation and DNA binding reported here, glucocorticoid mediated interference with the transcriptional machinery of
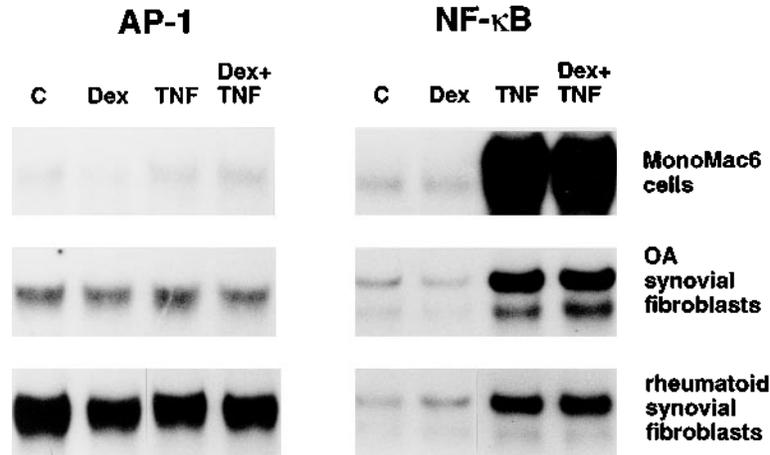
AP-1   NF-κB

Figure 8. Effects of dexamethasone and TNF on AP-1 and NF-κB DNA binding in cultured cells. Nuclear extracts prepared from Mono Mac 6 cells and from cultured OA and RA FLS were analyzed by EMSA. Cells were treated twice with dexamethasone 10^{-6} M or control for 18 h and 30 min and then stimulated with TNF 10 ng/ml for 30 min prior to harvest.

DNA-bound NF-κB is likely to contribute to the potent antiinflammatory effects of glucocorticoids[7]. It is therefore suggested that potent and specific inhibitors of NF-κB could have similar antiinflammatory efficacy as glucocorticoids.

Proinflammatory transcription factors NF-κB, AP-1, and NFAT are targets for the action of many antirheumatic drugs[38,39]. NF-κB is inhibited by glucocorticoids[1], gold thiolates[40], high dose aspirin[41], sulfasalazine[42], and leflunomide[43]. AP-1 is inhibited by glucocorticoids[1], gold thiolates[44], and D-penicillamine[45]. Nuclear factor of activated T cells (NFAT) is inhibited by cyclosporine[46]. The development of new antirheumatic agents that target transcription factors with greater specificity than these traditional drugs could include studies with fresh synovial tissue explants in order to obtain data that is relevant to human therapy.

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