

Adenoviral Gene Transfer of the Endogenous Inhibitor I κ B α into Human Osteoarthritis Synovial Fibroblasts Demonstrates That Several Matrix Metalloproteinases and Aggrecanases Are Nuclear Factor- κ B-Dependent

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ABSTRACT. *Objective.* To investigate the role of the transcription factor nuclear factor- κ B (NF- κ B) in promoting inflammatory and destructive responses in human osteoarthritis (OA) synovial fibroblasts, by assessing the effect of NF- κ B blockade on the production of cytokines and destructive enzymes.

Methods. Infection with adenoviruses transferring the β -galactosidase gene was used to ascertain that the OA fibroblasts could be infected (> 95%). Using an adenovirus transferring the inhibitory subunit I κ B α , effective inhibition of NF- κ B was achieved. The expression and production of several pro- and antiinflammatory cytokines and mediators, the major matrix metalloproteinases (MMP 1, 3, and 13), their main inhibitor tissue inhibitor of metalloproteinase-1 (TIMP-1), and the aggrecanases (ADAMTS4 and ADAMTS5) were measured by ELISA and/or reverse transcription-polymerase chain reaction, and their dependence on NF- κ B evaluated.

Results. The production of interleukin 6 (IL-6), monocyte chemoattractant protein-1, and RANTES was potently inhibited by I κ B α overexpression, irrespective of stimulus, but IL-8 was unaffected. The p55 soluble tumor necrosis factor (TNF) receptor was unaffected, but the p75 soluble TNF receptor was potently inhibited by I κ B α overexpression. MMP-1, MMP-3, and MMP-13 were inhibited by I κ B α overexpression, at both the mRNA and protein levels, whereas TIMP-1 mRNA was unaffected. The mRNA gene expression of ADAMTS4 was also inhibited by I κ B α overexpression, particularly in IL-1-stimulated cells, but ADAMTS5 was unaffected.

Conclusion. In OA synovial fibroblasts, inhibition of NF- κ B has a beneficial effect on the balance between the expression of proinflammatory cytokines and antiinflammatory mediators. Inhibition of this transcription factor also results in the decreased expression of several destructive metalloproteinases and also the ADAMTS4 aggrecanase. (First Release Jan 15 2007; J Rheumatol 2007;34:523–33)

Key Indexing Terms:

ADENOVIRUS
NUCLEAR FACTOR- κ B

AGGRECANASE

MATRIX METALLOPROTEINASE
OSTEOARTHRITIS

While there has been marked progress in the field of rheumatoid arthritis (RA), with advances in molecular pathogenesis leading to anti-tumor necrosis factor- α (anti-TNF- α) therapy gaining prominence^{1,2}, by comparison little is known about

the most common joint disease of all, osteoarthritis (OA). OA is complex, and neither its etiology nor its pathology is understood. In the majority of patients with OA, onset is spontaneous and not directly related to trauma or “wear and tear.” Further, there is a growing body of evidence that although cartilage degradation is the earliest event, synovial inflammation is implicated in many of the signs and symptoms of OA, including joint swelling and effusion^{3,4}. Synovitis in OA is not an innocent bystander, but contributes to disease progression, as judged by the correlation between biological markers of inflammation, such as C-reactive protein and cartilage oligomeric protein, with the progression of structural changes in OA^{5,6}. The overproduction of cytokines and growth factors from the inflamed synovium may play an important role in the pathophysiology of OA. Since the matrix metalloproteinases (MMP) have degradative effects on the extracellular matrix, some^{7,8} consider them important cofactors or disease mediators in OA. It is known that MMP-1 and MMP-13 are capable

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of cleaving collagen type II, and that MMP-3 is active against other components of the extracellular matrix, such as fibronectin and laminin. Although there has been some interest in MMP inhibitors as therapeutic agents in this disease⁹⁻¹¹, there is no clinical trial showing clear clinical benefit of such approaches, and hence the importance of these molecular pathways is not established.

Articular cartilage contains high concentrations of the large aggregating proteoglycan aggrecan. The high negative charge density of the glycosaminoglycan chains on aggrecan monomers in cartilage proteoglycan is essential for the ability of articular cartilage to withstand compressive deformation. The depletion of aggrecan from articular cartilage, as evidenced by the release of aggrecan catabolites into the synovial fluid, is a central pathophysiological event in OA. It has been shown that the release of aggrecan from both normal and OA cartilage involves a specific cleavage by a group of enzymes known as the aggrecanases, and that it does not involve the MMP^{12,13}. The aggrecanases are members of the family of disintegrin and metalloproteases with thrombospondin motifs (ADAMTS). To date, several such enzymes have been identified, among them aggrecanase-1 (ADAMTS4) and aggrecanase-2 (ADAMTS5). ADAMTS5 is constitutively expressed, but ADAMTS4 is induced following interleukin 1 (IL-1) or TNF treatment of cartilage explants¹⁴. In OA cartilage, aggrecanase activity and expression of ADAMTS4 and ADAMTS5 are present constitutively, with no requirement for any catabolic stimulation.

We used adenoviral gene transfer of the endogenous nuclear factor- κ B (NF- κ B) inhibitor I κ B α to study molecular pathways and potential therapeutic targets in OA fibroblasts. This technique is applicable in a large variety of cultured cells, and allows downregulation of one key signaling step, without the lack of specificity inherent in small-molecule inhibitors¹⁵⁻²⁰. Our specific aim was to study the role of NF- κ B on the balance of proinflammatory cytokines and antiinflammatory mediators, of destructive MMP and their inhibitor tissue inhibitor of metalloproteinase-1 (TIMP-1), and on aggrecanases, in a model of OA synovial fibroblasts stimulated with TNF- α , IL-1 β , or phorbol ester.

MATERIALS AND METHODS

Cells. Synovium from patients with OA undergoing joint surgery was cut into small pieces with sharp scissors, and digested with 1 mg/ml collagenase and DNase. Synovial fibroblasts were grown for 4–6 passages and then plated for adenoviral infection. Our study involved 18 patients in total, 16 women and 2 men, aged 45–83 years (median age 71 yrs). Ethical approval was obtained from the local ethics committees.

Adenoviral vectors. Recombinant adenoviral vectors encoding *E. coli* β -galactosidase (Adv β gal) or having no insert (Adv0) were provided by A. Byrnes and M. Wood (Oxford, UK). An adenovirus encoding porcine I κ B α with a cytomegalovirus promoter and a nuclear localization sequence (AdvI κ B α), and an adenovirus encoding the green fluorescent protein (AdvGFP), were provided by R. de Martin (Vienna, Austria). These were all first-generation, E1 and E3-deleted, serotype 5 adenoviruses. They were propagated in the 293 human embryonic kidney cell line and purified by

ultracentrifugation through 2 cesium chloride gradients²¹. The titers of viral stocks were determined through a plaque assay on 293 cells, as described²¹. All viruses used were plaque-purified from a master stock, in order to prevent contamination with wild-type adenovirus.

Analysis of infectibility. For infectibility experiments, cells were plated on 6-well plates in 0.5 ml serum-free RPMI-1640 at 0.5 million cells per well. After being allowed to adhere, they were either left uninfected, or infected with Adv β gal or Adv0 at a variable multiplicity of infection, to determine the optimal viral titer for later experiments. After 2 h, the supernatants were removed and replaced with 1.0 ml RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum. Cells were trypsinized off the plates 48 h after infection, spun down, and washed in fluorescence-activated cell-sorting (FACS) staining solution as described²². Each batch of uninfected, Adv0-infected, or Adv β gal-infected cells was then resuspended in 25 μ l of staining solution, and incubated at 37°C for 10 min, before 50 μ l of a 2 mM solution of fluorescein di- β -D-galactopyranoside (Sigma Chemical) was added for 1 min. Addition of excess (10 \times) ice-cold staining solution was used to stop the reaction. Cell fluorescence was analyzed by FACS as described²³. In a parallel series of experiments, infection of OA synovial cells with either Adv0 or AdvGFP was performed under the same conditions to verify the above findings, as judged by fluorescent microscopy 48 h after infection.

Western blotting. In these experiments, 3 batches of 5 \times 10⁶ cells each were either left uninfected or were infected with Adv0 or AdvI κ B α . Cytosolic and nuclear extracts were then prepared using extraction buffers and centrifugation as described²⁴ and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% (w/v) polyacrylamide gel, followed by electrotransfer onto nitrocellulose membranes. I κ B α and the p42/44 mitogen-activated protein kinases (MAPK) were detected using antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Electrophoretic mobility shift studies. Nuclear extracts (20 μ g) prepared as described above were mixed with 4 μ l 5 \times binding buffer prepared as described²², and the volume was brought up to 20 μ l with distilled H₂O. After 20 min at room temperature, 5 \times 10⁴ cpm of a double-stranded oligonucleotide probe for NF- κ B was added. After 20 min on a shaker, the resulting mixture was run on a pre-electrophoresed native (0.09 M Tris borate, 2 mM EDTA, pH 8.0) polyacrylamide gel for 90 min at 200 V. The gel was fixed, dried, and autoradiographed by exposure to Hyperfilm MP (Amersham GB).

Analysis of the production of cytokines and metalloproteinases. In experiments concerning cytokine and MMP production, 0.2 million OA fibroblasts were plated on a 12-well plate in 0.4 ml serum-free RPMI-1640. After being allowed to adhere, they were either left uninfected, or were infected with Adv0 or AdvI κ B α at a multiplicity of infection of 30:1, as suggested by previous infectibility experiments. After 2 h, the supernatants were removed and 1 ml RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum added. After 24 h, cells were stimulated with phorbol myristate acetate (PMA; 10 nM), IL-1 β (20 ng/ml), or TNF- α (20 ng/ml). After incubation for 48 h, the supernatants were taken off and analyzed for IL-6, IL-8, IL-11, M-CSF, granulocyte macrophage-colony stimulating factor (GM-CSF), oncostatin M, monocyte chemoattractant protein-1 (MCP-1), the IL-1 receptor antagonist, and the p55 and p75 soluble TNF receptors by ELISA (Table 1) using kits purchased from Amersham (Little Chalfont, Buckinghamshire, UK) and R & D Systems. The production of MMP-1, MMP-3, MMP-9, MMP-13, and TIMP-1 was analyzed by ELISA kits purchased from R & D Systems Inc. and from Amersham.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated from cell pellets as described²⁵ and isolated using TRIreagent (Sigma) and RNeasy (Qiagen Ltd., Crawley, UK) according to the manufacturer's protocol. RT-PCR analysis was performed using an RNA PCR kit (Perkin-Elmer, Warrington, UK) as described²⁶ using oligonucleotide primers corresponding to cDNA sequences for the MMP, TIMP-1, ADAMTS4, and ADAMTS5 (Table 2). Following an initial denaturation step of 1 min at 95°C, amplification consisted of between 30 and 60 cycles of 1 min at 95°C, 45 s at the primer annealing temperature, 30 s at 72°C, followed by a final extension step of 5 min at 72°C. PCR products were

Table 1. The effect of Adv0 and AdvIkB α infection on the production of cytokines and MMP, as assessed by ELISA. Mean (pg/ml) and SEM for each cytokine and MMP studied, in the 4 main categories (unstimulated cells, PMA-stimulated (10 nM) cells, IL-1 β -stimulated (20 ng/ml) cells, and TNF- α -stimulated (20 ng/ml) cells), each with cells left uninfected (Uninf), cells infected with Adv0 (30:1), and cells infected with AdvIkB α (30:1).

		Unstimulated			PMA			IL-1 β			TNF- α		
		Uninf	Adv0	AdvIkB α	Uninf	Adv0	AdvIkB α	Uninf	Adv0	AdvIkB α	Uninf	Adv0	AdvIkB α
IL-6 (n=6)	Mean	34761	32554	15986	132390	120129	31799	179029	153531	32426	226233	163571	28604
	SEM	6644	4241	2529	60878	53992	6860	61112	36160	16287	57736	74246	11112
IL-8 (n=7)	Mean	5529	3380	18163	16030	15790	29703	78109	72581	52768	96659	66863	52449
	SEM	1467	824	5112	3182	3633	4941	31664	15903	12139	27590	16494	11031
MCP (n=7)	Mean	6183	6353	1642	11618	11892	1214	30146	26805	1779	21165	29637	1636
	SEM	1420	1504	426	3730	2933	358	3432	6017	309	8481	10600	435
TNFR1 (n=6)	Mean	144	140	192	229	217	213	141	149	179	125	116	139
	SEM	24.7	29.6	56.2	64.7	62.8	67.5	44.5	51.4	69.0	22.8	23.3	47.7
TNFR2 (n=5)	Mean	32	20	6	106	118	10	45	36	3	35	25	6
	SEM	7.52	7.06	5.20	40.29	45.01	7.35	16.14	14.89	2.33	13.09	8.73	4.77
IL-11 (n=8)	Mean	244	240	177	4140	3929	3632	1611	1420	1729	2009	1807	1178
	SEM	86.2	90.7	43.8	1065.7	986.5	951.9	809.6	666.2	510.6	570.3	814.6	269.8
GM-CSF (n=6)	Mean	199	243	241	304	243	88	325	206	284	338	215	337
	SEM	193	242	219	176	188	42.8	180	110	216	159	129	206
RANTES (n=6)	Mean	51	38	20	71	62	20	616	405	26	724	868	26
	SEM	23.4	17.0	15.1	21.7	20.8	10.7	276.3	96.6	12.7	342.6	472.7	16.0
MMP-1 (n=7)	Mean	172819	196897	140708	297213	381592	153654	364571	319706	113432	230129	387235	60967
	SEM	112156	103820	80856	158644	276224	89896	213020	189024	58045	183045	308160	38205
MMP-3 (n=6)	Mean	59310	54897	50766	62433	64604	43469	136182	101347	39430	99764	70255	30615
	SEM	41120	39508	35839	42000	44483	29995	94621	68931	29202	64361	44364	21913
MMP-13 (n=6)	Mean	889	912	176	3036	2959	204	2068	1684	169	3343	2311	166
	SEM	515.13	546.99	65.78	1616.9	1462.3	79.25	797.3	600.7	82.36	1879.8	1246.6	81.28
TIMP-1 (n=8)	Mean	261721	118818	90848	380871	320909	262679	299476	344730	182405	322666	317417	171989
	SEM	77386	22605	13498	103121	122557	137374	119428	118974	107410	90590	113645	95630

Table 2. Oligonucleotide primers used for RT-PCR. Primer sequences correspond to sequences for human cDNAs deposited to GenBank. Where a mixed base is indicated (i.e., for GAPDH), the sequence also corresponds to the analogous rat cDNA.

Target Template	PCR Primers	Product Size (bp)	Annealing Temperature ($^{\circ}$ C)
GAPDH	5'TGG TAT CGT GGA AGG ACT CAT 5'GTG GGT GTC GCT GTT GAA GTC	370	53
ADAMTS4	5'GTC TGT GTC CAG GGC CGA TGC 5'GCC GCC GAA GGA TCT CCA GAA	541	61.8
ADAMTS5	5'GCG GAT GTG TGC AAG CTG ACC 5'AGT AGC CCA TGC CAT GCA GGA	487	57.4
MMP-1	5'ACA AAT CCC TTC TAC CCG GAA 5'GGA TCC ATA GAT CGT TTA TAT	314	50.8
MMP-3	5'CTT TTG GCG AAA ATC TCT CAG 5'AAA GAA ACC CAA ATG CTT CAA	404	50
MMP-13	5'TTC TGG CAC ACG CTT TTC CTC 5'GGT TGG GGT CTT CAT CTC CTG	273	53
TIMP-1	5'CCA CCT TAT ACC AGC GT TAT 5'CCT CAC AGC CAA CAG TGT AGG	282	54

visualized on a 3% agarose gel (containing 0.5 μ g/ml ethidium bromide) and their nucleotide sequences verified using an ABI-310 Genetic Analyser. PCR was routinely performed using different cycle numbers to avoid overamplification.

Analysis of apoptosis. To rule out that apoptosis-mediated cell depletion interfered with the results, cells were routinely checked by microscopy and through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. In addition, a series of experiments were performed using an assay for nuclear fragmentation²⁷, as follows: 0.5 million OA synovial cells were plat-

ed on 12-well plates and either left untreated or infected with 30:1 of Adv0 or AdvIkB α . Addition of cycloheximide (2 μ g/ml) and TNF- α (20 ng/ml) was used as a positive control. After 48 h, cells were stained 30 min in 1 ml hypotonic fluorochrome solution (50 μ g/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100) and the resulting propidium iodide-stained nuclei were analyzed by flow cytometry.

Caspase-3 is an intracellular protein that becomes activated during the cascade of events associated with apoptosis, making it suitable for analysis as an apoptosis marker²⁸. A colorimetric assay for caspase-3 enzymatic activity

was purchased from R & D Systems, and used according to the manufacturer's instructions.

Statistical testing. In all statistical testing, a one-sided, paired-comparisons Student's t test was used.

RESULTS

Efficient adenoviral gene transfer into OA fibroblasts. The infectibility of OA fibroblasts was investigated using the Adv β gal adenovirus. Since previous data^{16,19} indicated that RA synovial cells and human skin fibroblasts could be infected with 30–40:1 of adenovirus, titers in the range 10–100:1 were considered of interest. It was seen (Figure 1) that ~95% of OA fibroblasts were infected by a multiplicity of infection of 30 Adv β gal plaque-forming units per cell, and that increas-

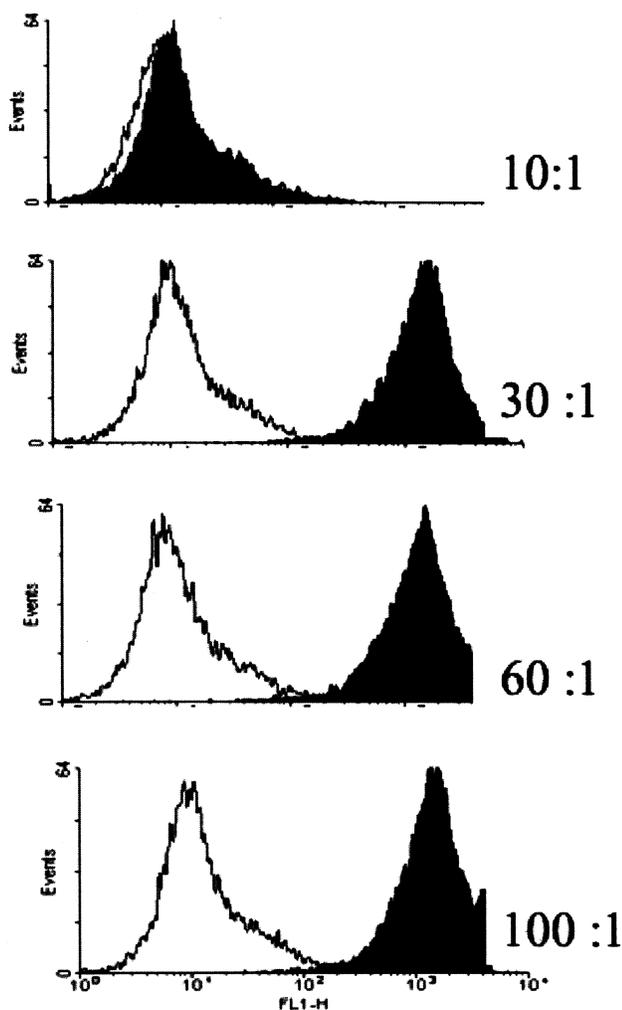


Figure 1. In excess of 95% of OA fibroblasts can be infected with adenovirus. OA fibroblasts were either left uninfected or were infected with various titers of an adenovirus with no insert (Adv0) or an adenovirus encoding *E. coli* β -galactosidase (Adv β gal). Cells were taken off the plate 48 h after infection, washed, and incubated in 37°C FACS staining solution for 10 min before addition of fluorescein-di-(β -D)-galactopyranoside for 1 min. Reaction was stopped by addition of 10 \times ice-cold staining solution, and cell fluorescence from Adv0-infected (black line) and Adv β gal-infected (black area) cells was analyzed by FACS. This figure represents 3 separate experiments.

ing the viral titer to 60:1 or 100:1 did not increase the percentage of infected cells. This finding was reproduced using AdvGFP, and it was observed that a titer of 30:1 infected the vast majority (> 95%) of OA fibroblasts (data not shown).

Analysis of I κ B α overexpression and NF- κ B function. Infection of OA fibroblasts with AdvI κ B α resulted in both cytosolic and nuclear overexpression of I κ B α , whereas infection with Adv0 had no such effect (Figure 2A). Stimulation of these fibroblasts with the phorbol ester PMA (or by TNF- α , data not shown) resulted in I κ B α degradation, but this did not affect the overexpressed I κ B α in AdvI κ B α -infected cells. Equivalent amounts of protein were loaded on each track, and reprobing with a p42/44 MAPK antibody was performed to demonstrate this and verify specificity. Stimulation of uninfected or Adv0-infected OA synovial fibroblasts with PMA, IL-1 β , or TNF- α resulted in increased NF- κ B activity on the electrophoretic mobility shift assay, but not in AdvI κ B α -infected cells (Figure 2B). This demonstrates that the AdvI κ B α adenovirus is functional in OA synovial cells, at a titer of 30:1. There was no consistent effect of Adv0 infection in these experiments, in either stimulated or unstimulated cells. Excess cold self versus scrambled oligonucleotide probes were routinely used to ascertain specificity.

Effect of I κ B α overexpression on cytokine production. Using either PMA, TNF- α , or IL-1 β as stimuli, the effect of I κ B α overexpression on the production of several pro- and anti-inflammatory cytokines was investigated in OA synovial fibroblasts. We observed that IL-6 protein production was very potently inhibited ($p < 0.001$) by I κ B α overexpression (80%; Figure 3A). Another cytokine that has been reported to be NF- κ B-dependent to a variable degree in fibroblast systems is IL-8, but in these OA fibroblasts, we could find no significant effect of I κ B α overexpression on the production of this cytokine, irrespective of stimulus (Figure 3B). In contrast, both MCP-1 and RANTES were strongly NF- κ B-dependent, irrespective of stimulus (Figure 3C, 3D). The production of GM-CSF induced by PMA or IL-1 was significantly inhibited ($p < 0.05$), but not the GM-CSF production induced by TNF (Figure 3E). There was no production of oncostatin M or M-CSF from these OA synovial fibroblasts, irrespective of stimulus (data not shown).

The OA fibroblasts also produce several antiinflammatory mediators in response to the stimuli listed above. There was no significant effect of I κ B α overexpression on IL-11 induced by PMA or IL-1, although the TNF response was significantly inhibited ($p < 0.05$; Figure 4A). There was also potent inhibition of the production of the p75 soluble TNF receptor, but no effect on the p55 receptor, irrespective of stimulus (Figure 4B, 4C). These OA fibroblasts did not produce measurable amounts of IL-10 or the IL-1 receptor antagonist (data not shown).

Effect of I κ B α overexpression on MMP and their main inhibitor TIMP-1. We observed that I κ B α overexpression

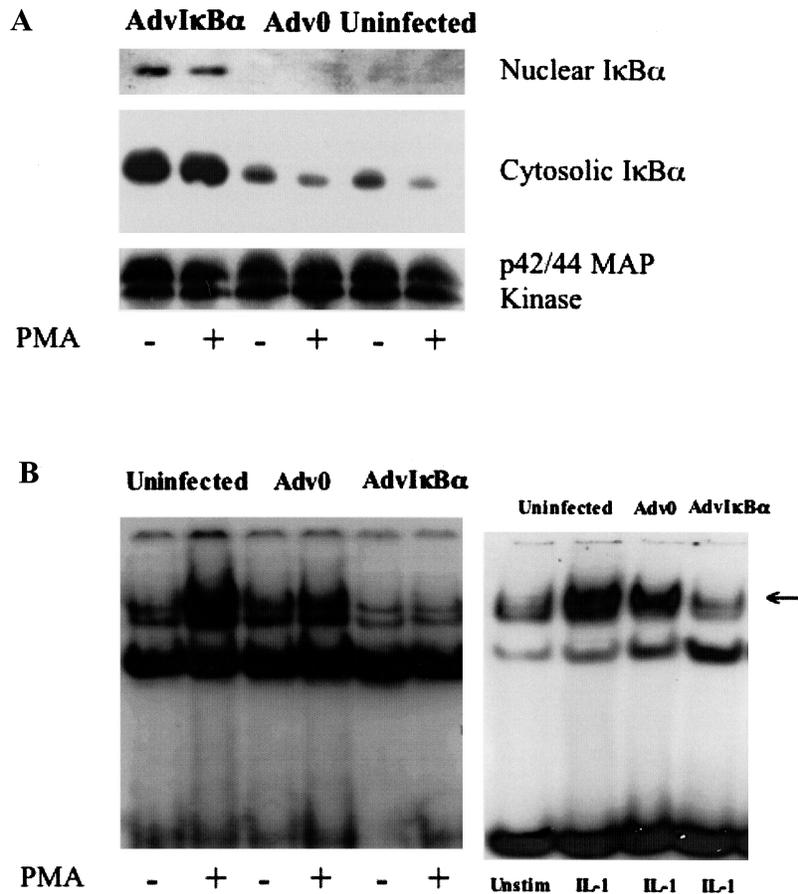


Figure 2. The AdvIκBα adenovirus is functional in OA. Fibroblasts infected with AdvIκBα (30:1), but not Adv0 (30:1), show nuclear and cytosolic overexpression of the IκBα molecule on Western blot analysis, while not affecting the p42/p44 MAPK used as a control (A). Nuclear extracts from the same experiments showed that 30:1 of AdvIκBα, but not Adv0, caused inhibition of PMA-induced (B, left panel) and IL-1β-induced (B, right panel) NF-κB activation on electrophoretic mobility shift assay. Arrow indicates NF-κB complexes.

potently inhibited ($p < 0.001$) the production of MMP-13 protein from OA synovial fibroblasts, irrespective of stimulus. The production of MMP-1 protein was also significantly inhibited ($p < 0.01$), irrespective of stimulus. In contrast, the production of MMP-3 induced by IL-1 or TNF was significantly inhibited ($p < 0.01$), but there was no significant effect on the PMA-induced response (Figure 5). The production of TIMP-1, the inducible inhibitor of these MMP, also showed stimulus specificity: the PMA-induced response was not significantly affected, but the TIMP-1 production induced by IL-1 or TNF was significantly inhibited ($p < 0.01$) by IκBα overexpression (Figure 5D).

RT-PCR analysis of gene expression of the MMP and TIMP showed inhibition of expression of MMP-1, MMP-3, and MMP-13 in AdvIκBα-infected cells (Figure 6). Again, there was more potent inhibition of MMP-13, irrespective of stimulus, indicating its strong dependence on NF-κB. With regard to MMP-3, the response to TNF was more potently

inhibited than that to PMA. The gene expression of TIMP-1 was not significantly affected by the overexpression of IκBα (Figure 6).

Effect of IκBα overexpression on the expression of aggrecanase. RT-PCR analysis of ADAMTS5 gene expression showed that this enzyme was only marginally induced by addition of PMA, IL-1, or TNF-α, and was unaffected by IκBα overexpression (Figure 7). In contrast, there was potent induction of ADAMTS4 gene expression by IL-1 and to some degree also by TNF-α. This induction of ADAMTS4 was potently inhibited by IκBα overexpression, whereas the constitutive level of the gene expression of this aggrecanase was unchanged (Figure 7). These experiments indicated a level of control at the NF-κB level in the ADAMTS4 gene, with regard to the upregulation that is induced by IL-1 or TNF-α.

PCR was routinely done using different cycle numbers to avoid overamplification. Each lane in these figures represents at least 3 experiments. In a series of 3 separate experiments,

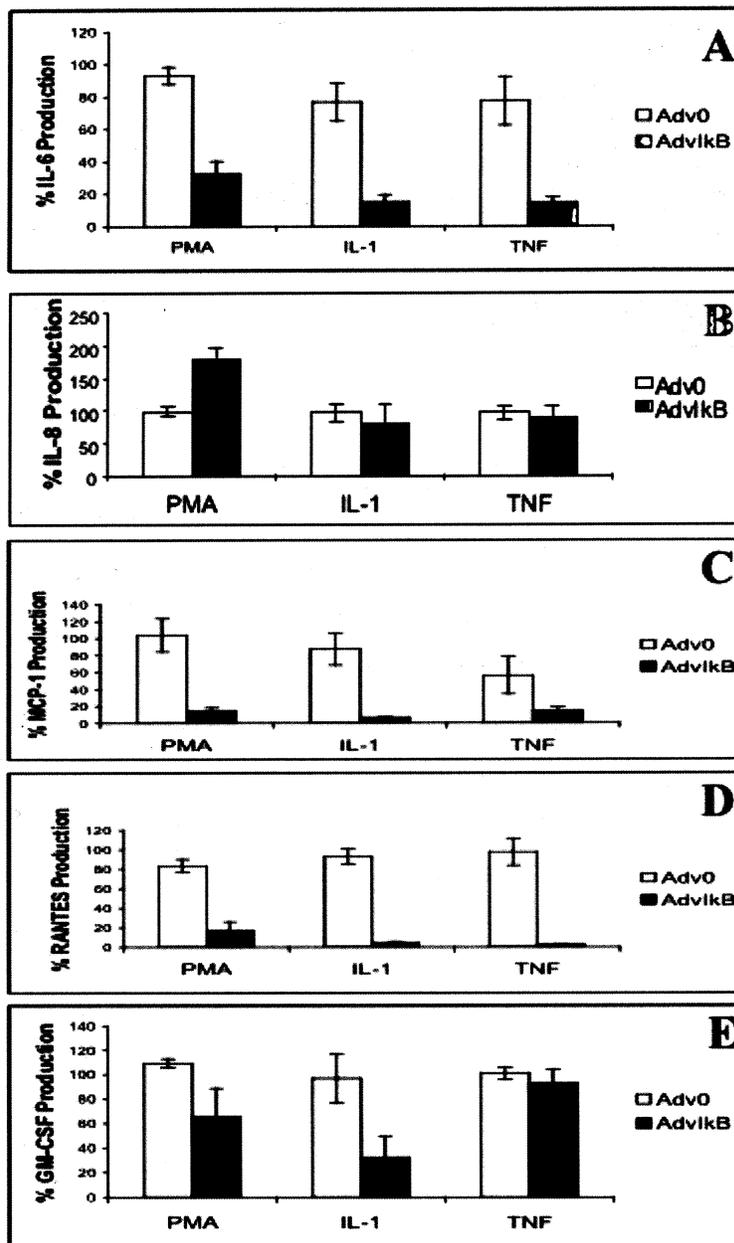


Figure 3. Effect of $\text{I}\kappa\text{B}\alpha$ overexpression on the production of proinflammatory cytokines from OA synovial fibroblasts. OA synovial fibroblasts were either left uninfected or were infected with 30:1 of either Adv $\text{I}\kappa\text{B}\alpha$ or Adv0, and the production of various proinflammatory cytokine proteins [IL-6 (A), IL-8 (B), MCP-1 (C), RANTES (D), and GM-CSF (E)], induced by PMA, TNF- α , or IL-1 β , was measured by ELISA (n = 7–10) and expressed as the percentage of the production from uninfected cells.

none showed any effect on the ADAMTS5 gene, whereas all 3 experiments showed consistent inhibition of ADAMTS4 (data not shown).

Analysis of apoptosis. Some studies have suggested that NF- κB inhibition may exert some of its effects by inducing apoptosis within the infected cell population^{29,30}. This is an important question to address to identify the mechanisms that lead to cytokine suppression and a reduction in MMP secretion,

and to rule out that the inhibition of various mediators is secondary to apoptosis-mediated cell depletion. In our study, several lines of evidence indicated that this was not the case. First, there was no reduction of cell numbers in Adv $\text{I}\kappa\text{B}\alpha$ -infected cultures compared with uninfected and Adv0-infected cells, nor was there any indication of increased cell death as judged by microscopy or the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Further, there was no

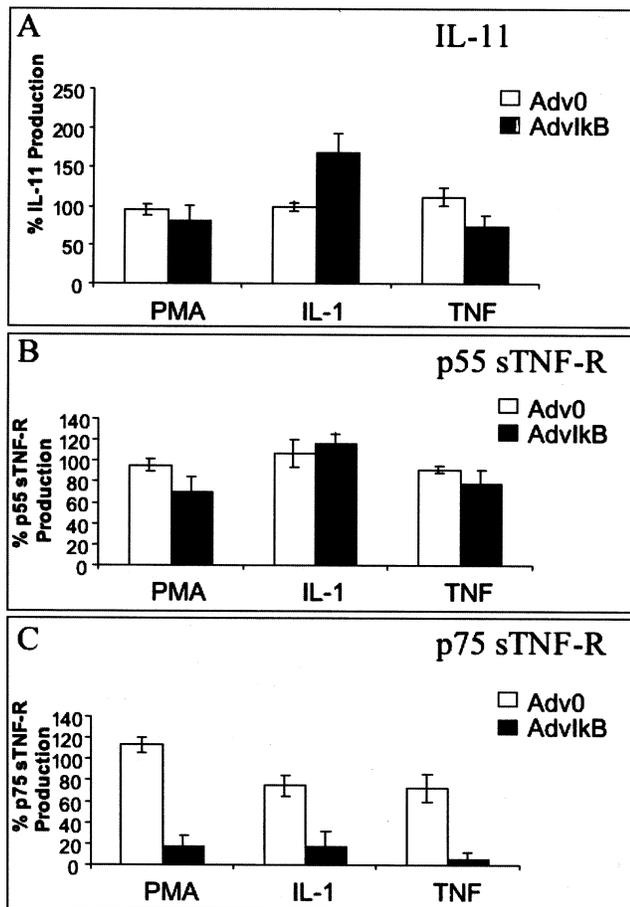


Figure 4. Effect of I κ B α overexpression on the production of proinflammatory cytokines from OA synovial fibroblasts. OA synovial fibroblasts were either left uninfected or were infected with 30:1 of either AdvI κ B α or Adv0, and the production of IL-11 (A), the p55 soluble TNF receptor (B), and the p75 soluble TNF receptor (C), induced by PMA, TNF- α , or IL-1 β , was measured by ELISA (n = 7–9) and expressed as the percentage of the production from uninfected cells.

reduction of the levels of the cytosolic control proteins (p42/44 MAPK; Figure 2A) or the mRNA levels of the house-keeping gene GADPH (Figure 6). Analysis of DNA fragmentation did not indicate that AdvI κ B α -infected fibroblasts underwent apoptosis, even when treated with TNF- α (data not shown), nor did an assay for Caspase 3 (Figure 8).

DISCUSSION

Adenoviral gene transfer is today widely recognized as a valuable tool to study intracellular signal transduction in various cell systems. There are several previous studies using adenoviral gene transfer in human skin fibroblasts, human lung fibroblasts, or RA synovial fibroblasts^{16,19,20,31} with a similar methodology and similar viral titers as those utilized here. In contrast, just a few studies have utilized OA synovial fibroblasts, mainly in the study of apoptosis^{32,33}. Due to fibroblast heterogeneity, we first demonstrated that adenoviral gene transfer is feasible in OA synovial fibroblasts, at a very

similar viral titer to what has previously been reported in other fibroblast models, and that an adenovirus transferring the I κ B α inhibitor of NF- κ B is functional in these cells. As verified by a combination of assays, gene transfer of I κ B α does not cause significant apoptosis in OA synovial fibroblasts. We chose to use 3 different stimuli in our study, namely the phorbol ester PMA, IL-1 β , and TNF- α . All 3 of these stimuli have previously been proven to induce both cytokines and MMP in other fibroblast models¹⁹. The reason to use these different stimuli was to pick up stimulus specificity in the regulation of these mediators, something that did not appear unlikely with regard to some of the MMP, due to their multifactorial regulation, with activating protein-1 (AP-1), NF- κ B, and other transcription factors involved^{34–36}.

Our finding that IL-6 is potently inhibited by I κ B α overexpression agrees with data from normal human macrophages and fibroblasts, as well as from RA and OA synovial cell cocultures, that IL-6 is a cytokine strongly dependent on transcriptional regulation by NF- κ B^{15,16,19}. In contrast, we found no effect of I κ B α overexpression on the production of IL-8, irrespective of stimulus. This is in disagreement with earlier published data from various fibroblast and smooth muscle cell systems^{16,19,37}, although it should be noted that the NF- κ B dependence of IL-8 is more pronounced in macrophages or in RA synovial cell cocultures than in human skin fibroblasts (compare Bondeson, *et al*¹⁵ and Bondeson, *et al*¹⁶ vs Bondeson, *et al*¹⁹). In the latter system, there was just 20–25% inhibition of IL-8 production induced by IL-1 or TNF¹⁹. There may be several pathways leading to IL-8 production, and the dependence on NF- κ B may be stronger in highly activated cells like freshly harvested RA synovial fibroblasts, than in resident fibroblasts stimulated with cytokines. In good agreement with earlier data^{28,38,39}, both MCP-1 and RANTES were strongly NF- κ B-dependent in OA synovial fibroblasts. With regard to GM-CSF, earlier results are contradictory^{28,40,41}, and there appears to be some degree of stimulus specificity in OA synovial fibroblasts, with the TNF response being NF- κ B-independent, whereas the response to IL-1 or PMA was NF- κ B-dependent. With regard to antiinflammatory mediators, a different picture emerges. Neither IL-11 nor the p55 soluble TNF receptor was NF- κ B-dependent, irrespective of stimulus. This agrees with earlier observations concerning these cytokines^{16,19,42}. It is a curious and novel finding that in OA synovial fibroblasts, there appeared to be differential regulation of the 2 soluble TNF receptors, the p75 soluble receptor being strongly NF- κ B-dependent, irrespective of stimulus, whereas the p55 soluble TNF receptor was unaffected by NF- κ B downregulation. A not dissimilar picture was observed in RA synovial cells¹⁶, although these cells seemed to produce less of the p55 soluble TNF receptor.

It is known that many MMP, including the important collagenases MMP-1 and MMP-13, are regulated through the interaction of several transcription factors, including AP-1, NF- κ B, and Ets-1^{34–36}. In RA¹⁶ or OA⁴³ synovial cell cocul-

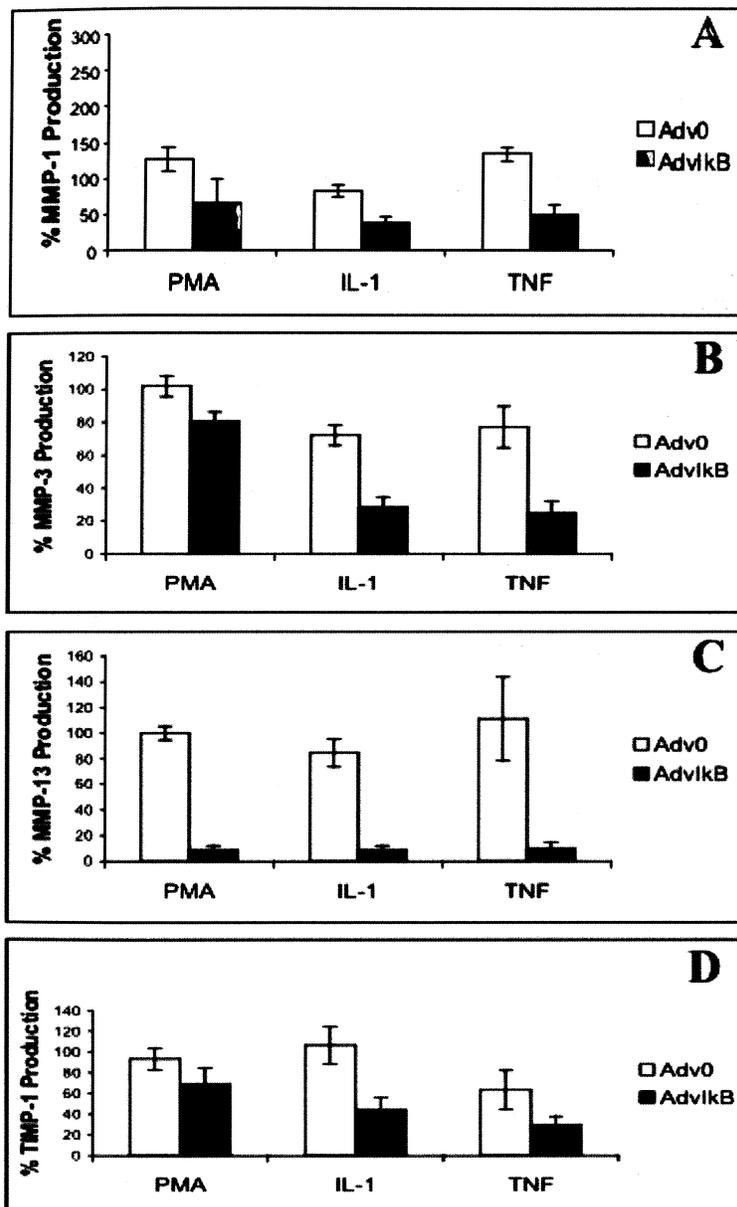


Figure 5. The production of several matrix metalloproteinases is NF- κ B dependent in OA fibroblasts. OA synovial fibroblasts were either left uninfected or infected with 30:1 of either AdvIkB α or Adv0, and the production of MMP-1 (A), MMP-3 (B), MMP-13 (C), and TIMP-1 (D) protein, induced by either PMA, TNF- α , or IL-1 β , was measured by ELISA (n = 6–9) and expressed as the percentage of the production from uninfected cells.

tures, MMP-1, 3, 9, and 13 are all NF- κ B-dependent, whereas TIMP-1 is not. Results from cultured fibroblasts have also provided evidence for these MMP being NF- κ B-dependent^{18,19,44}, although somewhat less potently than in the primary cells. In our OA synovial fibroblasts, MMP-13 was strongly NF- κ B-dependent on both the mRNA and the protein level (Figures 5 and 6). With regard to MMP-1 and MMP-3, there was some degree of stimulus specificity: the response to IL-1 or TNF was more potently inhibited by I κ B α overexpression than that to PMA (Figures 5 and 6). Although this

finding has theoretical interest with regard to the regulation of these MMP, its *in vivo* relevance is reduced by the fact that in OA synovial cell cocultures containing both synovial macrophages and synovial fibroblasts, both MMP-1 and MMP-3 are NF- κ B-dependent⁴³.

Ours is likely to be the first report to use adenoviral gene transfer to investigate the regulation of ADAMTS4 and ADAMTS5. Since the adenoviral infection does not lead to apoptosis, good-quality material was available for RT-PCR analysis of these enzymes. Results indicated that the basal

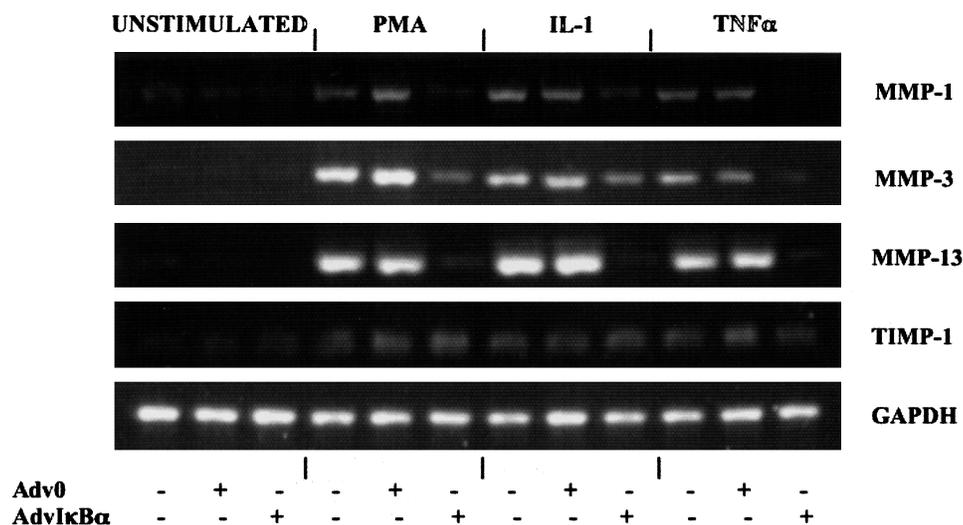


Figure 6. RT-PCR analysis of gene expression of MMP. OA synovial fibroblasts were either left uninfected or were infected with 30:1 of either AdvIkB α or Adv0. After 24 h, cells were stimulated with PMA, TNF- α , or IL-1 β . RT-PCR analysis was carried out using oligonucleotide primers specific for MMP-1, MMP-3, MMP-13, TIMP-1, and GAPDH for comparison of gene expression.

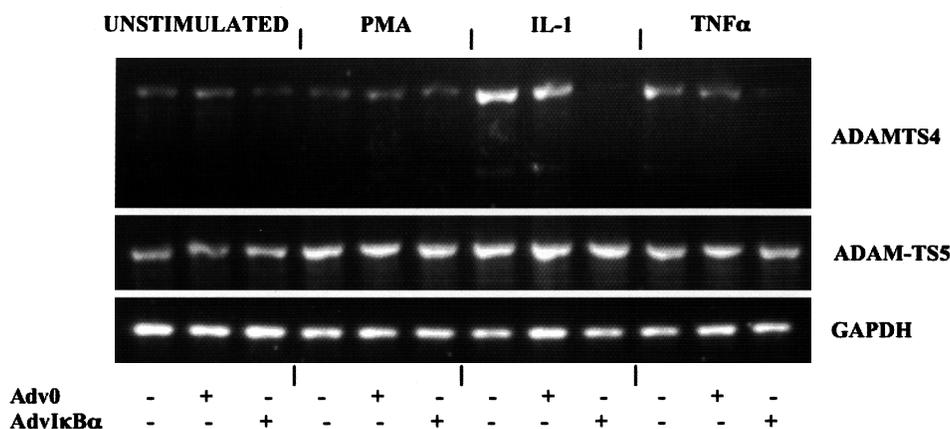


Figure 7. RT-PCR analysis of aggrecanase gene expression. OA synovial cells were either left uninfected or were infected with 30:1 of either AdvIkB α or Adv0. After 24 h, cells were stimulated with PMA, TNF- α , or IL-1 β . RT-PCR analysis was carried out using oligonucleotide primers specific for ADAMTS4 and ADAMTS5. Analysis of GAPDH gene expression was used for comparison.

level of both these aggrecanases was NF- κ B-independent in human OA fibroblasts. ADAMTS4, but not ADAMTS5, could be induced with either IL-1 or TNF- α . This response was strongly NF- κ B-dependent. Recent studies using transgenic mice⁴⁵⁻⁴⁷ suggest that in these murine models of degenerative joint disease, ADAMTS5 is the pathologically induced aggrecanase. However, our study in human OA synovial fibroblasts suggests that ADAMTS4 is the aggrecanase induced by proinflammatory cytokines in these cells, in a NF- κ B-dependent manner. The identification of the primary aggrecanase (ADAMTS4 or ADAMTS5) involved in human OA still needs to be conclusively established.

The overall effect of IkB α overexpression in OA synovial fibroblasts would appear to be a beneficial one. Several proinflammatory cytokines, including IL-6 and the chemokines MCP-1 and RANTES, are NF- κ B-dependent, but antiinflammatory mediators like the p55 soluble TNF receptor or IL-11 are not. Similarly, the major matrix metalloproteinases MMP-1, 3, and 13 are NF- κ B-dependent. An important and novel finding is that whereas the ADAMTS5 aggrecanase is NF- κ B-independent in OA synovial fibroblasts, the upregulation of the ADAMTS4 aggrecanase by IL-1 or TNF is NF- κ B-dependent in these cells.

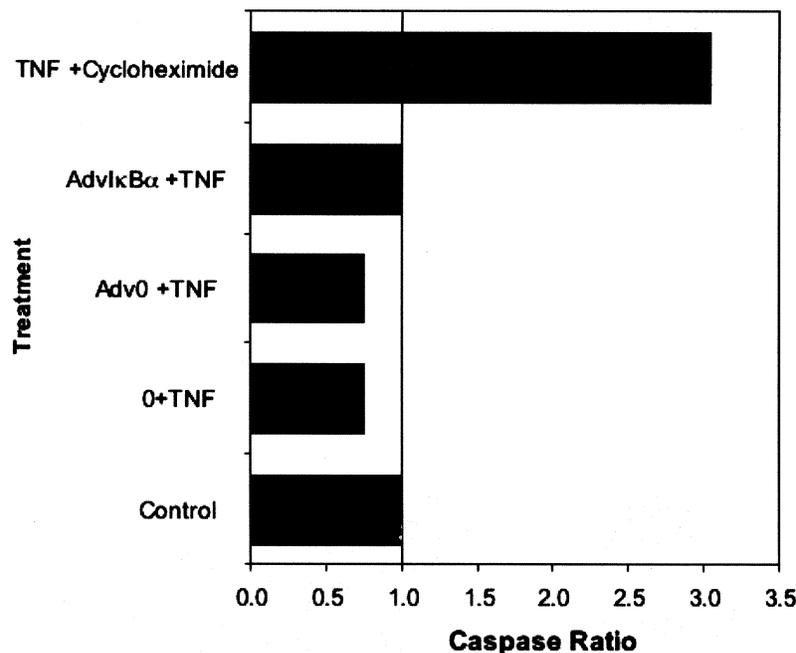


Figure 8. The AdvIkB α adenovirus does not cause apoptosis. One million OA synovial cells were plated on 12-well plates and either left untreated or infected with 30:1 of Adv0 or AdvIkB α , then were studied for caspase-3 activity, with addition of cycloheximide (2 μ g/ml) and TNF- α (20 ng/ml) for 16 h used as a positive control.

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