CONCISE REPORTS

Nuclear factor- κ B activity in T cells from patients with rheumatic diseases: A preliminary report

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Abstract

Objective—The NF- κ B/Rel family of transcription factors regulates the expression of many genes involved in the immune or inflammatory response at the transcriptional level. The aim of this study was to determine whether distinctive patterns of NF- κ B activation are seen in different forms of joint disease.

Methods—The DNA binding activity of these nucleoproteins was examined in purified synovial and peripheral T cells from patients with various chronic rheumatic diseases (12: four with rheumatoid arthritis; five with spondyloarthropathies; and three with osteoarthritis).

Results—Electrophoretic mobility shift assays disclosed two specific complexes bound to a NF- κ B specific 32 P-labelled oligonucleotide in nucleoproteins extracted from purified T cells isolated from synovial fluid and peripheral blood of patients with rheumatoid arthritis. The complexes consisted of p50/p50 homodimers and p50/p65 heterodimers. Increased NF- κ B binding to DNA in synovial T cells was observed relative to peripheral T cells. In non-rheumatoid arthritis, binding of NF- κ B in synovial T cells was exclusively mediated by p50/p50 homodimers.

Conclusion—Overall, the results suggest that NF- κ B may play a central part in the activation of infiltrating T cells in chronic rheumatoid arthritis. The activation of this nuclear factor is qualitatively different in rheumatoid synovial T cells to that in other forms of non-rheumatoid arthritis (for example, osteoarthritis, spondyloarthropathies).

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decameric sequences (κ B motifs) located in the promoter of many cellular and viral genes, particularly those encoding proteins involved in the immune and inflammatory response.^{3,4} NF- κ B is made of hetero- or homodimeric combinations of several proteins belonging to the same family (Rel family). The five known mammalian Rel/NF- κ B proteins, NF- κ B1 (p50), NF- κ B2 (p52), RelA (p65), RelB, and c-Rel, share a highly conserved Rel homology domain that contains sequences required for DNA binding, protein dimerisation, and nuclear localisation.⁵ The most common species are p50 subunit homodimers or heterodimers consisting of a p50 subunit and either a p65 (RelA) subunit or the product of the *c-rel* oncogene (c-Rel). In most cell types, NF- κ B is found in an inactive cytosolic form, retained by association with inhibitory proteins called I κ Bs. Upon cell activation by a vast number of agents, I κ B undergoes rapid phosphorylation in specific serine residues by the recently discovered I κ B kinases, IKK α and IKK β , (reviewed by Stancovski and Baltimore⁶) ubiquitination and degradation by the 26S proteasome pathway,⁷ then releasing the active NF- κ B to the nucleus.

To determine whether the pattern of NF- κ B activation is qualitatively different in rheumatoid arthritis (RA) compared with other rheumatic diseases and hence establish whether this transcription factor plays some part in the pathogenesis of different types of synovitis, we have examined the activation of NF- κ B in T cells obtained from peripheral blood (PT) and synovial fluid (ST) from patients with different types of arthritis, by studying the DNA binding of the NF- κ B/Rel transcription factor family in these cells. We have found a differential pattern of NF- κ B binding to DNA in T cells (ST and PT) from RA and non-RA patients, and the significance of this finding will be discussed.

Methods

PATIENTS

Twelve patients (table 1) with different types of synovitis were studied. Four were diagnosed with RA according to the American College of Rheumatology Criteria; five with spondyloarthropathies (Sp) according to the Amor and ESSG Criteria (two with reactive arthritis

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Nuclear transcription factors are proteins that bind to promoters and enhancers to stimulate (or, occasionally, inhibit) gene transcription through direct interaction with DNA.¹ Among those transcription factors, nuclear factor κ B (NF- κ B²) is one of the most extensively studied. This factor, formerly described as a κ enhancer binding transcription factor constitutively present in the nuclei of mature B cells,² regulates gene transcription by binding to

Table 1 Clinical data of patients studied

Patient	Sex	Age	Disease	RF	HLA-27	ANA	ESR	Hb (g/dl)	Treatment
1	F	58	RA	+	-	-	54	13	NSAID
2	M	69	RA	+	-	-	46	11.2	NSAID
3	F	32	RA	+	-	-	52	10.6	MTX
4	F	50	RA	+	-	-	45	11.8	NSAID
5	M	59	Spondyl/ReA	-	+	-	12	12	NSAID
6	M	14	Spondyl/ReA	-	+	-	19	14.2	—
7	M	32	Spondyl/AS	-	+	-	28	14.6	NSAID
8	M	59	Spondyl/AS	-	+	-	32	16.5	NSAID
9	F	25	Spondyl/AS	-	+	+ 1/300	13	14.6	NSAID
10	M	65	Osteoarthritis	-	-	-	2	11.8	—
11	M	50	Osteoarthritis	-	-	-	11	16.9	NSAID
12	M	57	Osteoarthritis	-	-	-	7	14.5	NSAID

RF = rheumatoid factor; B27 = HLA-B27; ANA = antinuclear antibodies; ESR = erythrocyte sedimentation rate; Hb = haemoglobin. Spondyl = spondyloarthropathy; AS = ankylosing spondylitis; ReA = reactive arthritis.

(ReA) and three with ankylosing spondylitis (AS)), and the remaining three with osteoarthritis (OA). Informed consent was obtained from all patients participating in this study.

ISOLATION OF PURIFIED T LYMPHOCYTES

Synovial fluid was collected by therapeutic knee arthrocentesis from all patients. Peripheral blood and synovial fluid were collected in heparinised tubes, and mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia, Piscataway, NJ, USA). Macrophages were depleted by adherence to plastic at 37°C for one hour, and B cells removed by passing the cells through a nylon wool column. In some experiments, the B cell fraction was removed by immunomagnetic separation using Dynabeads M-450 CD19 (Dyna, Oslo, Norway). The cell fraction obtained was usually >90% CD3⁺, as determined by immunofluorescence with specific mAbs.

PREPARATION OF PROTEIN EXTRACTS

Purified T cells from synovial or blood samples were centrifuged and transferred to Eppendorf tubes, and washed twice with cold phosphate buffered saline (PBS). Sedimented cells were gently resuspended and disrupted in 50 μ l of lysis buffer containing 20 mM Hepes pH 7.9, 0.35 M NaCl, 20% glycerol, 1 mM MgCl₂, 0.5 mM DTT, 0.5 mM EDTA, 0.1 mM EGTA, protease inhibitors (1 μ g/ml each of aprotinin, leupeptin, and pepstatin), 1 mM PMSF, and 0.2 % NP-40 at 4°C for 20 minutes. After lysis, samples were centrifuged at 10 000 $\times g$ for five minutes and supernatants carefully recovered. Protein concentration was determined by the Bradford method.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

A double stranded oligonucleotide containing the NF- κ B binding site located at the enhancer region of the H-2^b promoter (KBF) was used.⁸ The binding reagent contained 10 μ g of protein, 1 μ g poly(dI-dC), 20 mM Hepes (pH 7.0), 50 mM NaCl, 2 mM DTT, 0.01% NP-40, 100 μ g/ml BSA, 4% Ficoll, and 100 000 cpm of end labelled DNA fragments in a final volume of 20 μ l. Where indicated, 0.5 μ l of rabbit anti-p50, anti-p65, anti c-rel, anti-p52⁹ or preimmune serum was added to the reaction mixture before the addition of the radiolabelled probe. For cold competition, a

50-fold molar excess of the double stranded oligonucleotide competitor was added to the binding reaction medium. After 30 minutes incubation at room temperature, the mixture was electrophoresed through a 6% polyacrylamide gel under non-denaturing conditions and subsequently dried and exposed to XAR film at -80°C.

Results

NF- κ B binding to DNA in PT or ST cells from different patients was studied by gel retardation. An amount of 10 μ g of protein extract was incubated with a ³²P-labelled KBF oligonucleotide containing a high affinity binding site for NF- κ B.⁸ The experiments showed two distinct patterns of NF- κ B binding on the 12 patients studied.

PT cells from individual non-RA arthritic patients (n = 8) exhibited a single complex bound to the NF- κ B probe (fig 1A, complex II, AS, ReA). This protein/DNA binding pattern was similar to that found in proteins extracted from PT cells obtained from healthy donors (data not shown). By contrast, RA patients (n = 4) exhibited binding of two independent complexes, one with a migration pattern similar to that of the complex observed in non-RA patients (complex II) and the other, of lower mobility, present in both PT and ST cells (fig 1A, complex I, RA). DNA binding of both complexes was stronger in ST than in PT cells in all RA patients.

To demonstrate the specificity of the NF- κ B DNA binding pattern of figure 1A, several cold competition assays were conducted as described above. As can be seen in figure 1B, the binding activity to the KBF probe detected in ST cells was completely suppressed by a 50-fold molar excess of cold double stranded oligonucleotide containing the NF- κ B sequence of the Ig κ chain gene. On the other hand, binding was not hindered by an excess of unrelated oligonucleotides containing the consensus binding sites for OCT-1 and SP-1 transcription factors.

To identify the members of the NF- κ B/Rel family of transcription factors involved in the NF- κ B binding activity observed in ST cells from patients with different types of arthritis, supershift experiments with specific antisera were carried out. As can be seen in figure 1C (left), the protein/DNA complexes detected with the NF- κ B probe in ST cells (from a

Figure 1 (A) Nuclear factor κ B binding activity in proteins extracted from PT and ST cells belonging to four representative patients. AS, ankylosing spondylitis; ReA, reactive arthritis; RA, rheumatoid arthritis. Protein extracts were incubated with an end labelled specific κ B oligonucleotide and the specific DNA/protein interaction analysed by EMSA. A section of the gel is shown. (B) Specificity of NF- κ B binding activity in ST cells in three representative patients. Nuclear extracts of purified ST cells were incubated with 0.5 ng of an end labelled KBF probe. Competitor double stranded oligonucleotides were added at a 50-fold molar excess and included oligonucleotide Ig κ B, which contains the NF- κ B motif of the Ig κ gene, and the unrelated oligonucleotides OCT-1 and Sp-1. A section of the gel is shown. (C) Identification of NF- κ B complexes in ST cells from two representative patients. Protein extracts from purified T cells were preincubated either with preimmune serum (ns) or with specific antiserum for p50, p65 or p52, before the addition of KBF probe. A section of the gel is shown.

representative RA patient) were almost completely supershifted by anti-p50 antiserum. On the other hand, the anti-p65 antiserum prevented specifically the binding of the less mobile complex. Thus, the two main complexes detected in ST cells from RA patients were composed of p50/p50 homodimers and p50/RelA (p50/p65) heterodimers. In contrast, analysis of the NF- κ B DNA bound complex observed in non-RA arthritic patients showed it to consist exclusively of p50/p50 homodimers because of the presence of anti-p50, but

neither p65 or p52 antisera, in the binding reaction prevented the binding of such complex to the κ B probe (figure 1C, right).

Discussion

In this study, we found that the binding of NF- κ B to DNA was generally increased in purified synovial T cells from patients with various arthritic diseases when it was compared with peripheral T cells. The main inference from our results is the presence of p50/p65 heterodimers in PT and ST cells from RA patients, but not in those from other rheumatic diseases. These results show that NF- κ B activation is qualitatively different among the cases studied, possibly as a result of a differential pathogenesis for RA and other rheumatic diseases. The p50 subunit of the NF- κ B family does not have transcriptional activities over NF- κ B dependent promoters; rather, it may be inhibitory.¹⁰ Interestingly, an important pool of p50 proteins is located at the nucleus in non-transformed T cells,¹¹ but after activation by an appropriate stimulus, p50/p65 heterodimers translocate to the nucleus, bind the same NF- κ B sites as p50 homodimers, and regulate the transcription of many genes containing NF- κ B sites in their regulatory regions.¹⁰ In ST cells from RA patients, a specific stimulus may account for DNA binding of p50/p65 and may be mediated by specific antigen recognition or lymphokines such as tumour necrosis factor α and interleukin 1. This stimulus may be different in the other arthropathies studied, which only exhibited an increased binding of p50 homodimers. In this regard, it is interesting to note that, in addition to NF- κ B activation, the binding activity of AP-1 (another pleiotropic transcription factor) was only detected in ST cells from RA patients (data not shown).

Although our results were obtained from only 12 patients, these findings suggest that specific activation of NF- κ B (p50/p65) may play a pivotal part in the molecular mechanism involved in the pathogenesis of joint diseases in RA patients. Moreover, it supports the notion that T lymphocyte activation is different in patients with RA from that in patients with other rheumatic diseases as it has been previously suggested.^{12, 13} To confirm our finding that NF- κ B (p50/p65) activation in ST cells may be specifically related to RA patients, we are currently conducting a large scale study of patients with different types of arthritis.

Finally, it is clear that NF- κ B may be an attractive target for therapeutic intervention in RA and in other inflammatory diseases. Proteolytic degradation of I κ B and NF- κ B activation seems to be regulated by a balance between kinase and phosphatase activities, and the recent identification of two novel I κ B kinases involved in NF- κ B activation⁶ opens new avenues for anti-inflammatory drug design.

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- 1 Papavasilou AG. Transcription factors. *N Engl J Med* 1995;332:45–7.
- 2 Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 1991;46:705–14.
- 3 Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S. Rel/NF- κ B/I κ B family: intimate tales of association and dissociation. *Genes Dev* 1995;9:2723–35.
- 4 Finco TS, Baldwin Jr AS. Mechanistic aspects of NF- κ B regulation: the emerging role of phosphorylation and proteolysis. *Immunity* 1995;3:263–72.
- 5 Grilli M, Chiu JJ, Lenardo M. NF-kappa B and rel: participants in a multifactorial transcriptional regulatory system. *Int Rev Cytol* 1993;143:1–62.
- 6 Stancovski I, Baltimore D. NF- κ B activation. The I κ B kinase revealed? *Cell* 1997;91:299–302.
- 7 Traenckner EB-M, Wilk S, Baeuerle PA. A proteasome inhibitor prevents activation of NF- κ B and stabilises a newly phosphorylated form of I κ B α that it is still bound to NF- κ B. *EMBO J* 1994;13:5433–441.
- 8 Kieran M, Blank V, Logeat F, Vandekerckhove J, Lottspeich F, Le Bail O, *et al*. The DNA binding subunit of NF-kappa B is identical to factor KBF1 and homologous to the rel oncogene product. *Cell* 1990;62:1007–18.
- 9 Rice NR, Mackichan ML, Israël A. The precursor of NF-kappaB p50 has IkappaB-like functions. *Cell* 1992;71:243–53.
- 10 Plaksin D, Baeuerle PA, Eisenbach L. KBF1 (p50 NF- κ B homodimer) acts as a repressor of H-2K^b gene expression in metastatic tumor cells. *J Exp Med* 1993;177:1651–62.
- 11 Kang SM, Tran AC, Grilli M, Lenardo MJ. NF- κ B subunit regulation in non-transformed CD4⁺ T lymphocytes. *Science* 1992;256:1452–6.
- 12 Allen ME, Young SP, Michell RH, Bacon PA. Altered T-lymphocyte signaling in rheumatoid arthritis. *Eur J Immunol* 1995;25:1547–54.
- 13 Carruthers DM, Naylor WG, Allen ME, Kitas GD, Bacon PA, Young SP. Characterization of altered calcium signaling in T-lymphocytes from patients with rheumatoid arthritis (RA). *Clin Exp Immunol* 1996;105:291–6.