BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **220,** 120–124 (1996)
ARTICLE NO. 0367

Inducibility of RANTES mRNA by IL-1β in Human Bronchial Epithelial Cells Is Associated with Increased NF-κB DNA Binding Activity

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Received January 23, 1996

RANTES is a member of a large supergene family of proinflammatory chemokines that seems to play an important role in inflammatory processes. It is produced by many cell types in response to specific stimuli and during inflammatory reactions, but the marked differences in the pattern of induced expression suggest that different control mechanisms regulate transcription of RANTES in various tissue types. This is supported by the presence of a large number of potential binding sites for trascriptional factors in the promoter region of the RANTES gene. Our data indicate that expression of RANTES mRNA induced by IL-1β in human lung epithelial cells is associated with the activation of the transcriptional factor NF-κB.

One important requirement for the occurrence of inflammatory reactions at the tissue sites is the local release of mediators which attract and activate specific inflammatory cell populations. RANTES is a member of a new class of chemokines (1,2), that may play an important role in allergic inflammation because it exerts potent and selective chemotactic effects for cells which characteristically predominate in the allergic inflammatory infiltrates, namely CD45 RO^+ memory T lymphocytes (3), eosinophils (4,5) and mast cells (6). In addition, RANTES is produced by epithelial cells during eosinophil-rich inflammatory diseases (7).

Several cell populations can be induced to express RANTES gene and protein, including T lymphocytes (8), platelets (4), fibroblasts (9), renal epithelial cells (10) and lung epithelial cell lines (11,12), but the kinetic of RANTES expression is quite different. RANTES is expressed 3–5 days after activation of peripheral blood T lymphocytes with antigen and mitogen (8), whereas RANTES mRNA rapidly increases in fibroblasts and epithelial cells stimulated by TNFα and IL-1β (9–12). This suggests that different mechanisms may control RANTES transcription in various tissue types, and a better understanding of those mechanisms would provide new insight into the pathogenesis of certain inflammatory processes.

The 5′-flanking region of the RANTES gene contains a large number of potential binding sites for transcriptional factors (13). Some of these potential regulatory sites were originally described in promoters specifically expressed in cells like T lymphocytes, whereas other elements bind more ubiquitous factors, such as NF-κB, NF IL-6 and AP-1.

NF-κB is a trascriptional factor associated with rapid activation mechanisms. It is a heterodimer composed of structurally related DNA-binding subunits, p50 and p65 (14). In resting cells, NF-κB binding proteins are in an inactive cytoplasmic form, as they are complexed to members of a family of inhibitory proteins referred to as IκB. Extracellular signals which activate NF-κB initiate nuclear translocation and subsequent DNA binding of NF-κB by post-translational modifications resulting in the loss of inhibition by IκB proteins (15,16). As the promoter region of RANTES gene contains a large NF-κB site at position −32 (13), we examined whether NF-κB activation is involved in the induction of RANTES gene transcription in human airway epithelial cells.

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0006-291X/96 $18.00

MATERIALS AND METHODS

Epithelial cell cultures. Bronchial tissue was removed from 12 patients with lung cancer, who gave informed consent, at the time of surgical operation. After resection, macroscopically normal tissue fragments were taken as far away as possible from malignancy. Epithelium was stripped from the basement membrane under a dissecting microscope and epithelial cells were isolated as described (17–19). Their purity was greater than 98%, as assessed by immunospecific labelling (17–19). Cells were plated in collagen-coated 24-well plates and cultured in serum-free LHC-8 medium (Biofluids, Inc., Rockville, MD), supplemented with 100 U/mL penicillin G, 1 μg/mL fungizone and 1 μg/mL streptomycin. After an incubation period of 5–7 days, adherent epithelial cells were trypsinized, washed and replated at 10^4 cells per well in complete medium. Cells were cultured for additional 7 days at 37°C (5% CO_2 in air). Cells were trypsinized again and split 1:2 in 24-well plates. Semiconfluent cultures were obtained after 5 days of incubation. Epithelial cell monolayers were washed and incubated in medium devoid of growth factors (DME/F12 medium supplemented with antibiotics) 24 hours before use to make sure that the cells were in a quiescent stage at the time of testing (17–19). Then, increasing concentrations of recombinant human IL-1β (R&D Systems Europe, Oxon, UK) or the diluent alone were added to the culture wells, that were reincubated for 3–8, 12, 24, 36 and 48 hours. IL-1β stock solution and media contained undetectable levels of endotoxin (less than 50 pg/mL), as measured by the chromogenic LAL assay (Whitaker Bio Products, Walkersville, MD).

Northern blot analysis. To evaluate constitutive and induced expression of RANTES mRNA, RNA hybridization studies were carried out as previously described (17–19), with some modifications. Cytoplasmic RNA was extracted by the phenol-chloroform method and quantitated by absorbance at 260 nm. Samples were denatured (10 minutes at 65°C), size separated on agarose-formaldehyde gels (20 μg RNA/lane) and transblotted onto nylon membrane filters (ICN Biomedicals, Irvine, CA). The filters were dried, baked at 80°C for 2 hours, prehybridized for 24 hours at 42°C and hybridized overnight at 42°C with a ^{32}P-labelled RANTES DNA probe (1×10^6 cpm per mL). The prehybridization and hybridization fluids contained 3×SSC, 1% SDS, 50% formamide, 0.1 M sodium phosphate, 0.5 M sodium pyrophosphate (pH 7.0), 2.5×Denhardt's solution, 10 μg/mL salmon sperm DNA and 2.4% dextran sulfate. Filters were washed two times in 2×SSC/0.1% SDS for 30 minutes at room temperatures, and then in 0.2×SSC/0.1% SDS for 30 minutes at 60°C. Blots were dried and autoradiographed for 48 hours. The RANTES probe was an oligonucleotide synthesized on the basis of the published base-pair sequence of the coding region for human RANTES (8) (5′-GCTCATCTCGAAAGAGTTGATGTACTC-CCGAACCCATTTCTTCTCTGGGTT-3′). To check the quality and amounts of loaded RNA, the blots were rehybridized to a β-actin probe (17–19).

Electrophoretic mobility shift assay. Nuclear proteins were extracted from epithelial cells as described by Osborn and colleagues (20).

The DNA probe was a double stranded oligonucleotide containing the NF-κB binding site present in the RANTES promoter region (5′-ACT*CCCCTTAGGGGATGCCC*CTCA-3′) (13). The reagents for the binding reaction were 10 μg nuclear extract proteins and 1×10^5 cpm ^{32}P-labelled NF-κB probe in 15 μL of binding buffer (50 mM NaCl, 1 mM DTT, 10 mM Tris HCl, pH 7.5, 5% glycerol and 4 μg poly-dIdC). Reagents were incubated at 21°C for 30 minutes. The DNA-protein complexes were then resolved on a 5% polyacrylamide gel in 0.25×Tris borate at 150 V for 2 hours. After electrophoresis, gels were dried and DNA-protein complexes localized by autoradiography for 24 hours. Specificity of the binding was evaluated by adding 20-fold excess of unlabelled double-stranded oligonucleotide to the binding reaction 10 minutes before the addition of the labelled probe.

To identify the factors that bound to the NF-κB site, nuclear extracts from IL-1β-treated cells were incubated with 2 μL of a rabbit antiserum to p65, 2 μL of a rabbit antiserum to p50 (all from Santa Cruz Biotechnology, Le Perray en Yvelines, France) or 2 μL non-immune rabbit serum as control, for 20 minutes at 21°C, before addition of the binding buffer containing the labelled probe. Samples were subjected to electrophoresis as reported above.

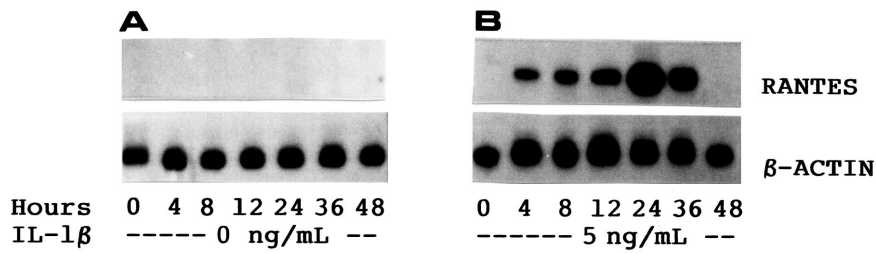

FIG. 1. Northern blot analysis showing RANTES mRNA expression in bronchial epithelial cells incubated for the indicated periods of time with the diluent of IL-1β (A) or with 5 ng/mL IL-1β (B). β-actin mRNA is also shown. (Each gel is representative of 5 experiments).

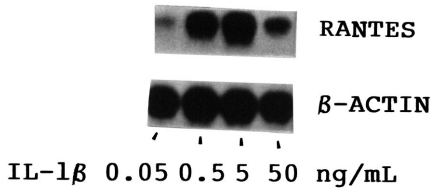

FIG. 2. Northern blot analysis showing the induction of RANTES mRNA in bronchial epithelial cells incubated for 24 hours with the indicated concentrations of IL-1β. β-actin mRNA is also reported. (Representative of 3 experiments).

RESULTS AND DISCUSSION

RANTES expression. Viability of the cells was greater than 90% in all the cultures tested, as demonstrated by the trypan blue exclusion method before RNA extraction.

Bronchial epithelial cells incubated with the diluent of IL-1β alone did not show RANTES transcripts at any of the time points tested (Fig. 1A), indicating that these cells do not express RANTES mRNA constitutively. By contrast, the epithelial cells exposed to 5 ng/mL IL-1β showed a time-dependent increase in RANTES mRNA expression in presence of comparable levels of the β-actin mRNA, which were consistent with equal loading of the lanes (Fig. 1B). The signal was detectable 4 hours after the addition of the stimulus to the culture media and peaked at 24 hours. Then, it decreased over the following 12 hours and disappeared by 48 hours. The Northern blot analysis showed hybridization bands of approximately 1250 bp, in keeping with the published data on human RANTES mRNA (8).

Inducibility of RANTES mRNA by IL-1β was dose-dependent, as shown in Figure 2.

NF-κB binding activity. IL-1β induced two specific NF-κB DNA binding complexes in epithelial cells (Fig. 3A), as demonstrated by competition with the unlabelled oligonucleotide (lane 6). A third complex also appeared, but it was not competed by the cold probe. Similar non-specific

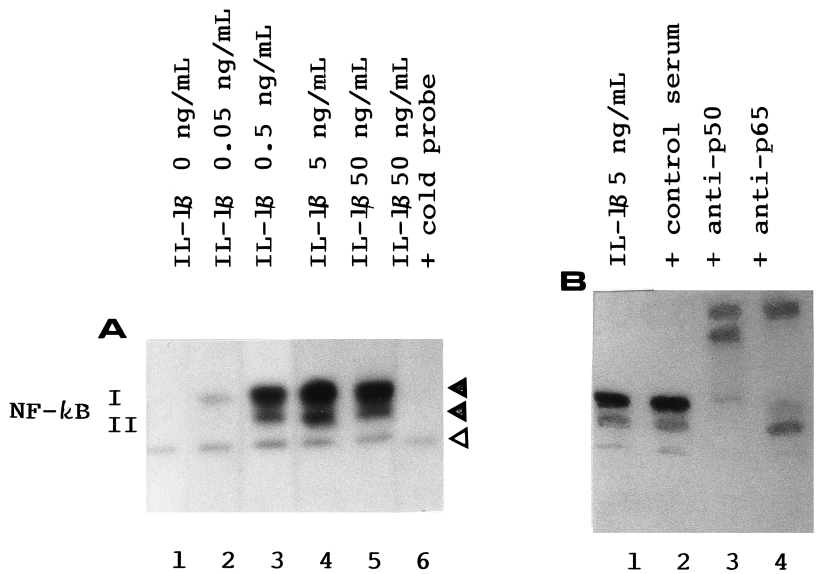

FIG. 3. Induction of NF-κB activity in bronchial epithelial cells (A) and effects of antisera on NF-κB DNA binding (B). Cells were incubated for 12 hours in presence or absence of IL-1β before extraction of the nuclear proteins for electrophoretic mobility shift assay. Specific and non-specific complexes are respectively indicated by filled and open arrowheads. (Each gel is representative of 5 experiments).

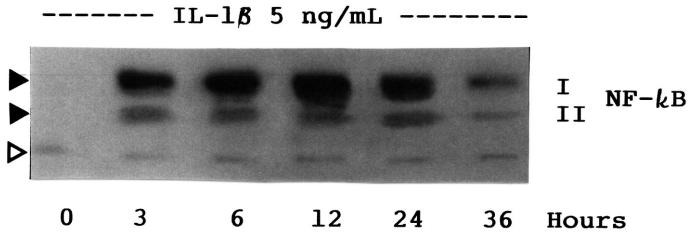

FIG. 4. Time course of the NF-κB binding activity in bronchial epithelial cells stimulated with 5 ng/mL IL-1β. (Representative of 5 experiments).

complexes have been previously observed in other cell types in response to stimuli which activate NF-κB (21,22).

To identify the proteins present in the RANTES NF-κB DNA complexes induced by IL-1β, epithelial cell nuclear extracts were incubated with antisera to p50 and p65 before electrophoretic mobility shift assay. Rabbit non-immune serum was used as control. The results are reported in Figure 3B. The non-immune serum had no effect on complex formation and mobility (lane 2). The antibody against p50 decreased the mobility of both induced complexes I and II, causing the supershift shown in lane 3. The antibody to p65 caused a supershift of complex I, but did not affect the mobility of complex II (lane 4). Thus, induced DNA-protein complexes did contain a p50-p65 heterodimer (complex I) and a p50-p50 homodimer (complex II). This definitely identified the factor that bound to the NF-κB site on the RANTES gene promoter as NF-κB.

The time course of induced NF-κB binding activity is shown in Figure 4. Nuclear NF-κB binding was detectable in epithelial cells 3 hours after the addition of IL-1β to the culture media, and it remained elevated for 36 hours. At this time, still there was transcription from the RANTES gene (Fig. 1B).

Our results indicate that NF-κB may be responsible for the temporally regulated pattern of RANTES gene expression induced by IL-1β in human bronchial epithelial cells.

ACKNOWLEDGMENTS

We thank Drs. S. Ancona and B. De Lellis, University of Milano, for providing the surgical material. This study was supported by the Italian National Research Council, Cariplo, and the Italian Foundation of Experimental Medicine.

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