Retinoids Inhibit Interleukin-12 Production in Macrophages through Physical Associations of Retinoid X Receptor and NFκB*

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Lipopolysaccharide (LPS) increases the production of interleukin-12 (IL-12) from mouse macrophages via a κB site within the IL-12 p40 promoter. In this study, we found that retinoids inhibit this LPS-stimulated production of IL-12 in a dose-dependent manner. The NFκB components p50 and p65 bound retinoid X receptor (RXR) in a ligand-independent manner in vitro, and the interaction interfaces involved the p50 residues 1-245, the p65 residues 194-441, and the N-terminal A/B/C domains of RXR. Activation of macrophages by LPS resulted in markedly enhanced binding activities to the κB site, which significantly decreased upon addition of retinoids, as demonstrated by the electrophoretic mobility shift assays. In cotransfections of CV-1 and HeLa cells, RXR also inhibited the NFκB transactivation in a ligand-dependent manner, whereas a mutant RXR lacking the AF2 transactivation domain, which serves as a ligand-independent binding site for transcription integrators SRC-1 and p300, was without any effect. In addition, coexpression of increasing amounts of SRC-1 or p300 relieved the retinoid-mediated inhibition of the NFκB transactivation. From these results, we propose that retinoid-mediated suppression of the IL-12 production from LPS-activated macrophages may involve both inhibition of the NFκB-DNA interactions and competitive recruitment of transcription integrators between NFκB and RXR.

Interleukin-12 (IL-12), 1 a heterodimeric cytokine composed of two disulfide-linked subunits of 35 (p35) and 40 (p40) kDa encoded by two separate genes, was originally identified in the supernatant fluid of Epstein-Barr virus-transformed human B cell lines (1, 2). IL-12 is produced by phagocytic cells and other antigen-presenting cells in response to stimulation by a variety of microorganisms as well as their products (3, 4). IL-12 exerts multiple biological activities mainly through T and natural killer cells by inducing their production of interferon-γ (IFN-γ), which in turn augments their cytotoxicity, and by enhancing their proliferative potential. IL-12 production is critical for the development of T helper type 1 (Th1) cells and the initiation of cell-mediated immune responses (reviewed in Ref. 5). The key role of IL-12 in inflammation as well as the cell-mediated immune responses (6, 7) have raised considerable interest in the mechanisms of IL-12 gene transcription. Inducible expression of IL-12 has been documented in macrophages and dendritic cells after stimulation by microbial antigens or via CD40-CD40L interaction (8, 9). In lipopolysaccharide (LPS)- and IFN-γ-treated monocytes, the expression of IL-12 p40 has been shown to be primarily regulated at the transcriptional level, which involved at least two transcription factors that belong to the NFκB and Ets families (10–12). Expression of IL-12 p35 is also known to be subject to similar transcriptional regulation, although characterized to a much lesser extent than p40 (13, 14).

The transcription factor NFκB is important for the inducible expression of a wide variety of cellular and viral genes (reviewed in Ref. 15). NFκB is composed of homo- and heterodimeric complexes of members of the Rel (NFκB) family of polypeptides. In vertebrates, this family comprises p50, p65 (RelA), c-Rel, p52, and RelB. These proteins share a 300-amino acid region, known as the Rel homology domain, which binds to DNA and mediates homo- and heterodimerization. This domain is also a target of the IκB inhibitors, which include IκBa, IκBβ, IκBγ, Bcl-3, p105, and p100 (16). In the majority of cells, NFκB exists in an inactive form in the cytoplasm, bound to the inhibitory IκB proteins. Treatment of cells with various inducers results in the degradation of IκB proteins. The bound NFκB is released and translocates to the nucleus, where it activates appropriate target genes.

The nuclear receptor superfamily is a group of ligand-dependent transcriptional regulatory proteins that function by binding to specific DNA sequences named hormone response elements in the promoters of target genes (reviewed in Ref. 17). The superfamily includes receptors for a variety of small hydrophobic ligands such as steroids, T3, and retinoids, as well as a large number of related proteins that do not have known ligands, referred to as orphan nuclear receptors. In particular, at least six distinct receptors have been extensively characterized for retinoids; retinoic acid receptor (RAR) α, β, and γ and retinoid X receptor (RXR) α, β, and γ (reviewed in Ref. 18). Functional analysis of nuclear receptors has shown that there are two major activation domains. The N-terminal domain (AF1) contains a ligand-independent activation function, whereas the extreme C-terminal region of the ligand binding
Inhibition of IL-12 Production by Retinoids

7675

domain (AF2) exhibits ligand-dependent transactivation and undergoes an allosteric change upon ligand binding. The AF2 region plays a critical role in mediating transactivation by a ligand-dependent interaction with transcription coactivators such as functionally conserved proteins CREB-binding protein (CBP) and p300 (reviewed in Ref. 19) and SRC-1 (20). Accordingly, deletion or point mutations in this region impair transcriptional activation without changing ligand and DNA binding affinities (21–23). CBP/p300 and SRC-1 have also been shown to be essential for the activation of transcription by a large number of regulated transcription factors, including CREB, NFκB, STATs, SRF, p53, and AP-1 (19, 24–27). Based on this broad spectrum of action, these coactivator proteins were termed transcription integrators.

Interestingly, members of steroid receptors including glucocorticoid receptor (28, 29), estrogen receptor (30, 31), progesterone receptor (32), and androgen receptor (33), have been shown to inhibit NFκB activity and can physically interact with NFκB in vitro. Since RelA represses ligand-dependent activation of steroid receptor-regulated promoters, a mutually inactive complex formed by a direct protein–protein interaction of steroid receptors and RelA has been proposed.

In this report, we found that retinoids inhibit the LPS-stimulated production of IL-12 from mouse macrophages through direct physical interactions of retinoid receptors with NFκB, like steroid receptors. The experimental results indicated that retinoid-mediated suppression of the IL-12 production from LPS-activated macrophages may involve both inhibition of the NFκB–DNA interactions and competitive recruitment of CBP/p300 and SRC-1 between NFκB and RXR.

EXPERIMENTAL PROCEDURES

Mice, Cell Lines, Culture Medium, and Transient Transfection—Female DBA/2 mice were obtained from Japan SLC, Inc. (Tokyo, Japan) and used at 6–10 weeks of age. RAW264.7 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO2 humidified air atmosphere. CV-1 cells as well as spleen cell populations and macrophages from mice were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS and antibiotics (Life Technologies, Inc.). For transfections, cells were grown in 24-well plates with medium supplemented with 10% FBS for 24 h and trans-fected with indicated plasmid in the presence of Superfect according to the manufacturer’s protocol (Qiagen). After 12 h, cells were washed and refed with DMEM containing 10% FBS. Cells were harvested 24 h later, luciferase activity was assayed as described (34), and the results were normalized to LacZ expression. Similar results were obtained in more than two separate experiments.

Monoclonal Antibodies (mAbs), Cytokines, and Reagents—Anti-IL-12 p40 mAbs C17.8 and C15.6 were purified from ascitic fluid by ammonium sulfate precipitation followed by DEAE-Sephadex chromatography (Sigma). Anti-IL-12 p35 mAb Red-TG297–289, anti-IL-10 mAbs JES-2A5 and SXC-1, as well as recombinant IL-12 p70, IL-10, and IL-12 p40 were purchased from R&D Systems, Inc. For transfections, mouse primary peritoneal macrophages from C57BL/6 mice were obtained as described previously (25–27).

Preparation of Splenic Macrophages Stimulated with LPS—Spleen cells were cultured at 10^6 cells/ml for approximately 3 h at 37 °C. The non-adherent cells were removed by washing with warm DMEM until visual inspection revealed a lack of lymphocytes (>98% of the cell population). The adherent cells were removed from plates by incubating for 15 min with ice-cold phosphate-buffered saline solution and rinsing repeatedly. The isolated adherent cell population was stimulated with 5 μg/ml LPS in the absence or presence of retinoids at 10−5, 10−4, and 10−3 M. At 1 × 10^6 cells/well, standard culture plates were incubated for 48 h.

Cytokine Assays—The quantities of IL-12 p40, IL-12 p70, and IL-10 in culture supernatants were determined by a sandwich enzyme-linked immunosorbent assay using mAbs specific for each cytokine, as described previously (36). The mAbs for coating the plates and the bioti-nylated second mAbs were as follows: for IL-12 p40, C17.8 and C15.6; for IL-12 p70, Red-TG297–289 and C17.8; for IL-10, JES-2A5 and SXC-1. Standard curves were generated using recombinant cytokines. The lower limit of detection was 30 pg/ml for IL-12 p40, 50 pg/ml for IL-12 p70, and 0.2 ng/ml for IL-10.

Electrophoretic Mobility Shift Assay—The nuclear extracts were prepared from the cells, as described previously (37). An oligonucleotide containing an NFκB-binding site within the Ig κ-chain (5’-CCG TTT AAG AGA GGG GGC TTT CCG AG-3’) was used as a probe. Labeled oligonucleotides (10,000 cpm) were incubated for 30 min at room temperature, along with 10 μg of nuclear extracts, in 20 μl of binding buffer (10 mM Tris HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng of poly(dI-dC), and 1 mM dithiothreitol). The reaction mixture was analyzed by electrophoresis on a 4% polyacrylamide gel in 0.5× Tris borate buffer. Specific binding was confirmed by competition experiments with a 50-fold excess of unlabeled, identical oligonucleotides or cAMP response element-containing oligonucleotides.

Statistical Analysis—Student’s t test was used to determine the statistical differences between various experimental and control groups. A p value of <0.01 was considered as significant.

RESULTS

Retinoids Inhibit IL-12 Production by LPS-activated Macrophages—We examined the effect of various retinoids including 9-cis-RA, TTNPB, and LG69 on the production of IL-12 by primary macrophages stimulated with LPS. 9-cis-RA is a pan-agonist for both RARs and RXRs, whereas TTNPB and LG69 are specific agonists for RARs and RXRs, respectively (38). LPS readily induced production of IL-12 heterodimer as well as the p40 subunit, as expected. However, retinoids inhibited this LPS-induced IL-12 production in a dose-dependent manner (Fig. 1). Interestingly, 9-cis-RA and LG69 were significantly more effective than TTNPB (p < 0.01 at 10−5 M RA). In contrast, treatment with retinoids did not influence IL-10 production from LPS-stimulated macrophages, suggesting that the retinoid effects were not the result of a general dampening of cellular activation.

Retinoids Inhibit NFκB-mediated Activation of IL-12 p40 Promoter by LPS—An IL-12 p40 subunit was known as the highly inducible and tightly regulated component of IL-12 (5). To identify the region involved in these retinoid actions, we generated a series of luciferase reporter constructs containing the p40 promoter sequences from positions −689, −231, and −185 to +98 relative to the transcription initiation site (Fig. 2A). Mouse RAW264.7 monocyteic cells were transfected with each of these constructs and stimulated with LPS either in the absence or presence of retinoids, and the luciferase activity was determined. All of these constructs showed strong stimulation with LPS in the absence of retinoids but impaired stimulation with retinoids (Fig. 2B). In particular, deleting sequences to −185 (p40/185) did not diminish the LPS-dependent promoter activities and the inhibitory effect of retinoids was still observed, suggesting that the target site for retinoids should reside within this region. To directly test the role of a κB site found between −121 and −131 of the p40 promoter in the retinoid-mediated inhibitory action, we introduced a linker scanning mutation into the κB site within the context of the −689/+98 construct (p40/LS). The LPS-dependent promoter activation was still observed with p40/LS, although significantly reduced (Fig. 2B), consistent with the previous findings in which the κB site was shown to be important for the LPS induction of p40 promoter (10). However, addition of retinoids.
to LPS-stimulated cells did not have any repressive effects with p40/LS, clearly indicating that the inhibitory effect of retinoids on IL-12 production was mediated through the NFκB site.

Physical Interaction of Retinoid Receptors with NFκB—With the precedent of direct physical interactions of NFκB with steroid receptors (28–33), we hypothesized that associations of NFκB with retinoid receptors may have led to the NFκB-inhibitory action of retinoids. Indeed, in vitro translated, labeled RXR interacted with GST fusions to the NFκB components p50 and p65 but not with GST alone, in a ligand-independent manner (Fig. 3A). Similarly, RXRAAP2, deleted for the C-terminal AF2 domain (39), and RXR-ABC containing only the N-terminal A/B/C domains also interacted with GST fusions to p50 and p65. In contrast, RXR-LBD, which contains only the hinge and ligand binding domains, did not bind any of these GST proteins. The RXR interaction domains were also mapped to the p50 residues 1–245 and the p65 residues 194–441 by using a series of C-terminal deletions introduced into p50 and p65 (Fig. 3B). Similarly, RAR also interacted with the p50 residues 1–245 and the p65 residues 194–441 (data not shown).

NFκB Binding to the κB Site Inhibited by Retinoid—Steroid receptors have been shown to inhibit NFκB binding to κB sites in a ligand-dependent manner (28–33). To examine whether retinoid-mediated inhibition of the NFκB transactivation also exploits similar mechanisms, we analyzed the κB binding activity present in nuclear extract of unstimulated or LPS-stimulated primary macrophages, either in the absence or presence of 10–7 M retinoids. As expected, nuclear extracts from LPS-stimulated macrophages exhibited strong κB binding activity in the electrophoretic mobility shift assays using a labeled oligonucleotide containing a consensus Ig-κB site (40) (Fig. 4A). The binding was specific as it was competed with an unlabeled, identical oligonucleotide, but not with unrelated, nonspecific oligonu-
cleted, and was absent with nuclear extracts from nonstimulated cells. Similar to steroid receptors, nuclear extracts from macrophages stimulated by LPS in the presence of various retinoids showed much diminished kB binding activities in a retinoid-dose-dependent manner (Fig. 4A). To rule out the possibility that these inhibitory actions of retinoids are the results of retinoid-directed gene expressions of a third component, retinoids were directly added to the binding reactions, along with nuclear extracts from LPS-stimulated macrophages. In these experiments, the kB binding activities decreased in a retinoid-dose-dependent manner, suggesting that the retinoid-bound receptor may directly modulate the NFkB-DNA interactions by forming a complex with NFkB that is unable to bind kB sites (Fig. 4B).

An Inhibitory Complex of NFkB-RXR—To test if this retinoid-mediated inhibition of NFkB activities in macrophages are generally observed in other cell types, we employed a reporter construct kB-LUC, previously characterized to efficiently mediate the NFkB-dependent transactivations in various cell types, that consists of a minimal promoter from the IL-2 gene and four upstream kB sites from the IL-2 gene (41). Cotransfection of CV-1 cells with RXR had a minimal effect on the kB binding activities in macrophages. In the presence of retinoids, however, increasing amounts of cotransfected RXR inhibited the reporter gene expression in an RXR dose-dependent manner (Fig. 5A). Similar results were also obtained with the LPS- or TNFα-induced level of transactivations in various cell types, including HeLa cells (data not shown). Similarly, cotransfection of increasing amounts of p50 or p65 also inhibited the 9-cis-RA-dependent transactivation by RXR (Fig. 5B). These results suggest that the interactions of NFkB-RXR may lead to a formation of transcriptionally inactive complex in vivo, regardless of the nature of DNA binding sites.

The C-terminal AF2 Domain Is Involved with NFkB Inhibition—Interestingly, cotransfection of CV-1 cells with RXRΔAF2, a mutant RXR lacking the C-terminal AF2 domain, moderately enhanced the reporter gene expression in the absence of retinoids, whereas it appeared unable to inhibit the NFkB transactivation in the presence of 9-cis-RA (Fig. 6A). The AF2 domain serves as ligand-dependent binding sites for a number of transcription coactivator molecules such as SRC-1 (20) and CBP (19), which were also shown to function as transcription coactivators for NFκB (25, 42, 43). Thus, competition for limiting amounts of SRC-1 and p300 could account for the mutual inhibitions between NFκB and liganded RXR. Indeed, the inhibitory effects of NFkB by liganded RXR were largely relieved upon addition of increasing amounts of SRC-1 and p300 expression vectors (Fig. 6B). Similar results were also obtained with NFkB-mediated inhibition of the RXR transactivation (data not shown).

The NFkB-inhibitory Actions of Retinoids Independent of kB Sites—Since the RXR binding sites involved the DNA binding Rel domain of both p50 and p65 (Fig. 3), we tested whether the NFkB-inhibitory actions of retinoids require kB site bindings. Thus, we expressed a Gal4 fusion protein to p65 (Gal4/p65) in CV-1 cells, along with a reporter construct controlled by upstream Gal4 sites (35). Consistent with previous findings (44), Gal4/p65 directed a strong activation of the reporter gene expression (Fig. 7). Cotransfection of increasing amount of RXR expression vector was without any significant effects in the absence of retinoids. In contrast, however, liganded RXR directed inhibition of the Gal4/p65 transactivation in an RXR dose-dependent manner (Fig. 7). These results, along with the results shown in Fig. 6, suggest that the inhibitory actions of retinoids can also operate without kB site bindings and involve the AF2-dependent factors.

DISCUSSION

Inhibiting the action of IL-12 has been shown to prevent development and progression of disease in experimental models of autoimmunity (45). These findings have raised great interest in identifying inhibitors of IL-12 production for the treatment of Th1-mediated diseases such as type-1 diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and acute graft-versus-host disease. Recently, corticosteroids have been shown to enhance the capacity of macrophages to induce IL-4 synthesis in CD4+ T cells by inhibiting IL-12 production (46). In addition, captopril and lisinopril, angiotensin-converting enzyme inhibitors, were also shown to suppress IL-12 production from human peripheral blood mononuclear cells (47). Phosphodiesterase inhibitor pentoxifylline...
Interestingly, 1,25-dihydroxyvitamin D3 was also shown to inhibit IL-12 production from human monocytes or dendritic cells by increasing intracellular cAMP levels, leading to inhibition of the development of Th1 cells while promoting Th2 cell differentiation (50). In this report, we added retinoids to the list of hydrophobic compounds including salbutamol inhibited IL-12 production from human monocytes or dendritic cells by increasing intracellular cAMP levels, leading to inhibition of the development of Th1 cells while promoting Th2 cell differentiation (50). Interestingly, 1,25-dihydroxyvitamin D3 was also shown to inhibit IL-12 production, presumably by down-regulating the NFκB activities from human IL-12 p40 gene (51).

In this report, we added retinoids to the list of hydrophobic compounds that inhibit production of IL-12 through specific nuclear receptors (17), together with corticosteroids (46) and 1,25-dihydroxyvitamin D3 (51) (Fig. 1). As was the case with corticosteroids and 1,25-dihydroxyvitamin D3, this inhibition was also mapped to a region in the p40 promoter containing a binding site for NFκB (Fig. 2) and may involve direct physical interactions of retinoid receptors with NFκB (Fig. 3). However, it is interesting to note that NFκB constitutively interacted with RXR (Fig. 3), whereas the inhibitory actions were absolutely ligand-dependent (Figs. 1, 2, and 5). Thus, NFκB may exist constitutively associated with RXR in vivo, and this complex becomes transcriptionally inactive upon addition of retinoids, in which ligand-dependent interactions with transcription coactivators may play important roles (as summarized in Fig. 8). This notion is consistent with the inability of the AF2-mutant RXR to inhibit the NFκB transactivation as well as derepression of the inhibitory actions by coexpressed SRC-1 and p300 (Fig. 6). However, it is not certain whether these coactivators remain complexed with NFκB in the presence of liganded RXR. In addition, retinoids also inhibited the NFκB transactivation as well as derepression of the inhibitory actions by coexpressed SRC-1 and p300 (Fig. 8). This notion is consistent with the inability of the AF2-mutant RXR to inhibit the NFκB transactivation as well as derepression of the inhibitory actions by coexpressed SRC-1 and p300 (Fig. 6). However, it is not certain whether these coactivators remain complexed with NFκB in the presence of liganded RXR. In addition, retinoids also inhibited the NFκB binding activities of NFκB in vitro (Fig. 4), suggesting that the liganded RXR/NFκB complex is unable to recognize NFκB sites. However, it is not currently known why this liganded RXR/NFκB complex loses its ability to bind NFκB sites. It is possible that conformational change brought into this complex, along with the transcription coactivators SRC-1 and CBP/p300, upon addition of retinoids may become propagated to the Rel homology domain of NFκB, resulting in inability to bind NFκB sites. However, the inhibitory actions of retinoids can also operate in the absence of NFκB in vivo, as demonstrated by the results shown in Fig. 7, in which transactivation mediated by Gal4/p65 was shown to be inhibited by retinoids. Overall, these results are similar to previously described results with steroid receptors (28–33), in which the mutual inhibitions be-
Inhibition of IL-12 Production by Retinoids

The retinoid receptor-NFκB interactions are likely to have wide implications in various aspects of retinoid and NFκB biology, not limited to the regulation of IL-12 production in macrophages described in this study. Both retinoids and NFκB have been shown to be involved with a wide variety of biological processes, including immunomodulation, embryonic development, spermatogenesis, and inhibition of cancer cell proliferation (reviewed in Ref. 52). In particular, this negative cross-talk could be relevant for tumor inhibitory actions of retinoids.

In conclusion, we have shown that retinoid receptors form a transcriptionally inhibitory complex with NFκB. With the NFκB transactivation, in particular, this retinoid-mediated inhibitory action appeared to involve inhibition of the NFκB-DNA interactions as well as competitive recruitment of transcription integrators between NFκB and RXR. This transrepression between NFκB and retinoid receptors could play an important role in a large variety of biological processes.

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Inhibition of IL-12 Production by Retinoids

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