Retinoic Acid Receptor-Nuclear Factor-Interleukin 6 Antagonism

A NOVEL MECHANISM OF RETINOID-DEPENDENT INHIBITION OF A KERATINOCYTE HYPERPROLIFERATIVE DIFFERENTIATION MARKER

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Retinoids inhibit the expression of migration inhibitory factor-related protein-8 (MRP-8), a marker of hyperproliferative or abnormal keratinocyte differentiation, in a retinoic acid receptor (RAR)-dependent manner in various cell culture systems. MRP-8 expression is also down-regulated in vivo in psoriatic lesions after topical application of an anti-psoriatic RAR 3γ-selective synthetic retinoid, tazarotene. We demonstrate that an MRP-8 promoter linked to a chloramphenicol acetyltransferase reporter (MRP8CAT) faithfully replicates the differentiation-specific regulation of the endogenous keratinocyte MRP-8 gene. Further, interferon γ and serum-induced expression of MRP8CAT is inhibited by retinoid receptors in a ligand-dependent manner. We also show that NF-IL6 acts as a transcriptional enhancer of MRP-8, and that RARs inhibit MRP8CAT by inhibiting the enhancer action of nuclear factor-interleukin-6 (NF-IL6). The NF-IL6 antagonism function of RAR is a complex of the core of the DNA binding domain and the hydrophobic zipper region. This manuscript identifies NF-IL6 as another transcription factor, in addition to AP1, whose activity is inhibited by RAR in a ligand-dependent manner. The interdiction of NF-IL6-dependent signal transduction pathway by RARs may explain some of the therapeutic effects of retinoids in inflammatory and proliferative diseases.

All-trans-retinoic acid (RA)1 and its synthetic analogs not only modulate cell growth and differentiation in vitro, but are also therapeutically effective in the treatment of a variety of diseases involving cell proliferation, abnormal differentiation, and inflammation (1, 2). The pleiotropic effects of retinoids are mediated through two families of nuclear retinoid receptors, retinoic acid receptors (RARα, β, and γ) and retinoid X receptors (RXRs, β, and γ). RARs and RXRs are modular proteins containing domains responsible for sequence-specific DNA binding (C-region), ligand independent transactivation (AF-1, A/B region), and ligand-dependent transactivation (AF-2, E-region) (1, 3). Retinoid receptors transactivate the expression of genes in a ligand-dependent manner by sequence-specific binding to the retinoic acid-responsive elements (RAREs) present in the promoter regions of responsive genes. In contrast to the steroid receptors, which function as homodimers, retinoid receptors function inside the cells as RAR-RXR heterodimers (4). Although the retinoid-dependent transactivation of genes can thus be explained by the binding of RAR-RXR to the RAREs, recruitment of co-activators, and subsequent activation of the transcriptional machinery, this paradigm does not hold true for the genes whose expression is repressed by retinoids. While RARs have been shown to inhibit the expression of certain genes by antagonizing the enhancer actions of the transcription factor AP1 (5, 6), the mechanism of retinoid-mediated inhibition of other non-AP1-dependent genes remains unclear. Since various inflammatory and hyperproliferative conditions appear to be maintained or perpetuated by the products of genes which are under the control of an AP1 motif, the antagonism of AP1-dependent gene expression appears to be an important underlying mechanism of the therapeutic action of RA.

Tazarotene is an RAR 3γ-selective synthetic retinoid which is therapeutically effective in the treatment of psoriasis (6), a highly prevalent skin disease characterized by hyperproliferation and inflammation (7). To understand the mechanism of tazarotene action in psoriasis, we have identified several tazarotene-responsive genes in an in vitro skin system by subtractive hybridization (8, 9). The expression of one of the genes, migration inhibitory factor-related protein-8 (MRP-8), was inhibited in an RAR-dependent manner in vitro in cultured keratinocytes and in skin raft organ cultures, as well as in vivo in psoriatic lesions (9). MRP-8 is highly expressed in psoriatic epidermis, while its expression is absent in normal epidermis (10). MRP-8 is also expressed in other chronic inflammatory diseases such as chronic polyarthritis and cystic fibrosis and displays a 73% amino acid homology to a murine cytokine CP-10 (11–14).

In this manuscript we demonstrate that retinoids inhibit the expression of MRP-8 by directly acting at the promoter level. We also show that the MRP-8 promoter is activated by NF-IL6 and retinoids inhibit MRP-8 expression by antagonizing the enhancer action of NF-IL6. We further demonstrate that the integrity of the core of the DNA binding domain (DBD) and the hydrophobic zipper region of RAR is mandatory for the inhibition of the MRP-8 promoter, thus indicating that the NF-IL6 antagonism domain of RAR is a complex of the DBD and the hydrophobic zipper region.

MATERIALS AND METHODS

Recombinant Plasmids—The cloning and sequencing of MRP-8 and its promoter has been previously reported (15). MRP8CAT was prepared by generating PCR primers to sequences approximately 1490 bp from the TATA box (5′-AGAAAGCTTCACCCTTTT7GCTT-3′) and to the reported sequences for the MRP-8 promoter just 5′ of the TATA box.
As in the case of the endogenous MRP-8 gene, treatment of HeLa cells with retinoids reduced NF-IL6 binding sites were found in the promoter at approximately 0.1–0.2 μg of RAR expression vector, except where noted, along with 2 μg of LipofectAMINE (Life Technologies, Inc.) for each well in a total volume of 0.5 ml. Retinoids were added 18 h post-transfection. HaCaT cells and primary human keratinocytes were plated at 50% confluence in 12 well plates and transfected the next day by either the poly-L-ornithine method previously described (19) with a 3-min 25% dimethyl sulfoxide shock, or by the TransIT-LT1 polyamine transfection reagent following the manufacturer’s instructions (PanVera Corp., Madison, WI) and using 2 μl of reagent per μg of DNA with a 4-h incubation period. For NF-IL6-induced expression of MRP8CAT, NF-IL6 expression vector was co-transfected with MRP8CAT in HaCaT cells, and CAT activity was assayed 36 h later. For retinoid inhibition of NF-IL6-induced MRP8CAT activity, 1.8 μg of MRP8CAT was transfected per well along with 0.2 μg of NF-IL6 and 0.5 μg of RAR expression vectors. Retinoids were added immediately after transfection, and CAT activity was assayed 36 h later. For inhibition of serum-induced activity by NF-IL6 antisense, MRP8CAT was co-transfected along with NF-IL6-AS in HaCaTs, and the cells were switched into KGM containing 10% delipidated serum 18 h later. CAT activity was assayed 48 h after the serum was added.

### RESULTS

**Construction of the MRP8CAT Reporter**—It has been demonstrated that the serum and interferon-γ (IFN-γ) induced expression of MRP-8 is repressed by retinoids in vitro both in cultured keratinocytes and skin raft cultures (9). The constitutively high levels of MRP-8 expression in patient psoriatic lesions is also down-regulated by treatment with tazarotene, an anti-psoriatic retinoid (9). To delineate the mechanism of retinoid action on the MRP-8 gene, a reporter was generated containing 1.5 kb of the MRP-8 promoter sequence linked to a chloramphenicol acetyltransferase (CAT) gene (MRP8CAT, Fig. 1a). A computer analysis of the cloned MRP-8 promoter sequence (Mac DNAisis) for transcription factor binding motifs did not reveal a canonical RARE. Surprisingly, perfect AP1 consensus sites were also not observed (AP1 is the only transcription factor to date shown to be antagonized by RARs). A variant AP1 site (5’-TGACTAA-3’) was found –465 bp upstream from the TATA box (Fig. 1b). In the context of MRP-8 and HSV-tk promoters however, this site was found to be inactive to TPA stimulation both in keratinocytes and HeLa cells (data not shown). These results suggested that RARs may be inhibiting the expression of MRP8CAT by antagonizing the activity of a non-AP1 transcription factor. Interestingly, three NF-κB binding sites were found in the promoter at -390 bp to -487 to -495 bp, and -692 to -700 bp from the TATA box (Fig. 1b). NF-IL6, like jun/fos, belongs to the bZIP family of transcription factors (20).

**The Expression of MRP8CAT Is Inhibited by RARs in a Ligand-dependent Manner**—Primary human keratinocytes were transiently transfected with MRP8CAT and treated with either 10% charcoal-treated serum (Fig. 2a) or 1000 units/ml IFN-γ (Fig. 2b). As in the case of the endogenous MRP-8 gene, treatment of keratinocytes with either of these differentiating agents stimulated expression of MRP8CAT and resulted in approximately 4–7.5-fold induction of CAT activity (Fig. 2, a and b). The serum and IFN-γ-induced expression of MRP8CAT was completely blocked, however, by co-treatment of primary keratinocytes with tazarotene (1 μM) (Fig. 2, a and b). A transformed keratinocyte cell line (HaCaT) was also transfected with the reporter and induction of MRP8CAT assayed after treatment of the cells with 10% charcoal-treated serum. Like the primary keratinocytes, serum treatment in HaCaT cells caused an induction of MRP8CAT (Fig. 2c). Interestingly, co-treatment of the HaCaT cells with retinoids did not result in a significant reduction of this activity (Fig. 2c). This result is most likely due to low levels of endogenous RAR expression in HaCaT cells, which is common for transformed cells. However, co-transfection with RARα, β, or γ expression vectors was able to repress the serum-induced expression of MRP8CAT in HaCaT cells after treatment with tazarotene (1 μM) (Fig. 2c). The activity of MRP8CAT was also checked in a HeLa cell line. Surprisingly, high basal levels of MRP8CAT expression were detected in HeLa cells cultured in Dulbecco’s modified Eagle’s medium with serum (10%). This high level of expression could be further stimulated by the addition of IFN-γ (1000 units/ml), and this stimulation could be blocked by tazarotene (Fig. 2d). In all experiments, the high basal expression of MRP8CAT was stimulated 25–45% by the addition of IFN-γ. As in keratinocytes, the AP1-like site was not responsive to TPA treatment (Fig. 2d). Further, both the high basal expression as well as the IFN-γ-stimulated expression of MRP8CAT could be repressed by the co-transfection of RARα, β, and γ expression plasmids in the presence of tazarotene (1 μM) (Fig. 2, d and e). The expression of MRP8CAT in HeLa cells could also be repressed in a dose-dependent manner in the presence of co-transfected RARγ by the RAR agonist tazarotene, AGN 190121, and TTNPB (Fig. 2, f). Like tazarotene, AGN 190121 is a RARγ-selective retinoid, while TTNPB is a pan-agonist for all RARs (6).

**RARs Inhibit MRP8CAT Expression by Antagonizing the Effect of Transcription Factor NF-IL6**—As stated, a computer analysis of the MRP-8 promoter revealed the presence of three NF-IL6 consensus sequences. To determine if this bZIP transcription factor could activate the MRP8CAT construct, an expression vector for NF-IL6 was co-transfected into HaCaT
cells along with MRP8CAT. Co-transfection of 0.1, 0.5, and 1.0 μg of NF-IL6 expression vector induced MRP8CAT expression (Fig. 3a). To determine if retinoids could inhibit the NF-IL6-induced expression, HaCaT cells were co-transfected with the MRP8CAT reporter and expression vectors for NF-IL6 and RARs in the presence or absence of retinoid. As previously shown for the serum induction of MRP8CAT, retinoids were unable to inhibit the induction by NF-IL6 in the absence of co-transfected RARs, suggesting that the available pool of RARs in the HaCaT cells is insufficient for trans-repression (data not shown). However, when an expression vector for RARγ was co-transfected along with the NF-IL6 expression vector, the activity of MRP8CAT was repressed in a dose-dependent manner after the treatment of the cells with tazarotene or TTNFB (Fig. 3b). This shows that like AP1, RARs are able to inhibit the induction by NF-IL6 in the presence of co-transfected RARs (Fig. 3b). The regions of the DBD and the hydrophobic zipper—RARγD and RARγC, which deletes the entire C region, and RARγCys1, which destroys the first zinc finger—were not able to repress MRP8CAT activity. In contrast, mutants of the DBD (RARα Cys1, which destroys the first zinc finger) were able to inhibit the expression of MRP8CAT in the presence of TTNFB. Therefore, the A/B regions and the ligand independent transactivation function AF-1 of RAR are not required for anti-NF-IL6 activity. In addition, mutants of the DBD (RARα Cys1, which destroys the first zinc finger) were not able to repress MRP8CAT activity. In contrast, mutants of the DBD (RARα Cys1, which destroys the first zinc finger) were able to inhibit the expression of MRP8CAT in the presence of TTNFB. Therefore, the A/B regions and the ligand independent transactivation function AF-1 of RAR are not required for anti-NF-IL6 activity. In addition, a mutation of the ligand binding domain (dnRARα), which binds ligand in vitro (21), also failed to inhibit MRP8CAT activity. Finally, two hydrophobic zipper mutants (RARαL328P and RARαM377R) were tested. RARαL328P has a leucine to proline substitution at amino acid position 328 which disrupts the 4th and 5th heptad repeats of the hydrophobic zipper, and M377R contains a methionine to arginine substitution at position 377. RARαL328P binds ligand but fails
to heterodimerize with RXR (18), whereas RAR-M377R binds ligand and acts as a dominant negative mutant in transactivation assays. Neither of these mutants was effective in inhibiting MRP8CAT activity. Interestingly, the same mutations to the DBD and the heterodimerization domain that interfere with RAR-anti-AP1 interaction also interfered with RAR-anti-NF-IL6 interaction, suggesting that similar mechanisms are involved (Fig. 4). Thus, the anti-AP1 domain, which consists of a core of the DBD and the heterodimerization domain, also functions as the anti-NF-IL6 domain.

We have recently demonstrated that MRP-8 expression is inhibited by retinoids in vitro in skin systems such as cultured keratinocytes and skin rafts. Further, we have demonstrated that the topical treatment of psoriatic plaques with the anti-psoriatic retinoid tazarotene inhibits the expression of MRP-8 in psoriatic lesions during the course of clinical improvement of the disease (9). To delineate the mechanism of negative regulation of MRP-8 by retinoids, the promoter region of MRP-8 was cloned into a reporter plasmid and analyzed by transient transfection. The expression of MRP8CAT was induced in primary keratinocytes, a keratinocyte cell line (HaCaT), and HeLa cells, thus indicating that the elements involved in the serum and IFN-γ induction of the endogenous MRP-8 gene are present in the cloned upstream sequences. Further, both serum- and IFN-γ-induced expression of MRP8CAT was inhibited by retinoids in keratinocytes and in HeLa cells (Fig. 2). These results demonstrate that the 1.5 kb of cloned MRP-8 promoter also harbors the retinoid regulatory sequences responsible for the repression of the gene. A motif search of the MRP-8 promoter did not turn up any obvious RAREs. An AP1-like motif (5'‐TGACTAA-3') present at -2465 bp from the TATA box, however, was inactive since the MRP8CAT construct was non-responsive to TPA induction in both cultured keratinocytes and HeLa cells (Fig. 2). A search of the MRP-8 promoter revealed the presence of three putative NF-IL6 binding motifs. Co-transfection experiments with MRP8CAT and an NF-IL6 expression vector showed that NF-IL6 induced the MRP8CAT reporter (Fig. 3). NF-IL6, like c-Jun/c-Fos, is a bZip transcription factor and belongs to the family of C/EBPs (20). RAR inhibited the NF-IL6-induced expression of MRP8CAT in a ligand-dependent manner (Fig. 3). NF-IL6 is the second transcription factor identified so far whose enhancer action, like that of AP1, is antagonized by retinoids. Using various mutants of RARs, we demonstrate that the integrity of the DBD as well as the hydrophobic zipper region is obligatory for inhibition of MRP-8 gene expression (Fig. 4). The dispensability of the A/B regions, together with the requirement of the core of the DBD and the intact hydrophobic zipper suggested that the NF-IL6 antagonism function of RAR is a complex of two distinct domains (Fig. 4). Note that tazarotene, which is an RARβ/γ-
selective retinoid in transactivation assays, inhibited the expression of the MRP-8 promoter through RARα (Fig. 2). Therefore, like the anti-AP1 property of RARα (6), the anti-NF-IL6 activity of RARα appears to be separable from its transactivation function.

Retinoids are widely accepted as therapeutic modalities for the treatment of dermatological diseases including psoriasis and have been shown to be effective in the treatment of various cancers (e.g. acute promyelocytic leukemia, squamous cell carcinoma, Kaposi’s sarcoma, etc.). Further, retinoids inhibit the proliferation of a number of other cancer cell lines (e.g. breast, ovarian, colon etc.) in vitro (1). Although activation of genes has been thought to be the major mode of action of RARs, so far only one retinoid-induced gene has been identified which can transduce the anti-proliferative and anti-inflammatory signals of retinoids. Insulin-like growth factor-binding protein 3 is induced by retinoids in transformed cervical cells and inhibits the growth-promoting activities of insulin-like growth factor 1 (22). In contrast, a number of retinoid-repressed genes are associated with therapeutic effects of retinoids (6). The antagonism of AP1-dependent gene expression can explain some of the therapeutic effects of retinoids in various proliferative and inflammatory diseases. Since NF-IL6 is another pro-inflammatory and proliferative transcription factor, the inhibition of its activity delineates another pathway for the therapeutic effects of retinoids. NF-IL6 regulates the expression of IL-6 (23), and as shown here, MRP-8, both of which are highly expressed in psoriasis (10, 24) and involved in the pathophysiology of the disease. Therefore, by antagonizing NF-IL6 action, retinoids would inhibit both IL-6 and MRP-8 in psoriatic lesions, resulting in disease amelioration. Retinoids, in fact, inhibit the expression of IL-6 in cultured keratinocytes and skin rafts. Further, RAR-NF-IL6 antagonism can also account for the normalization of abnormal keratinocyte differentiation, since MRP-8 is associated with the abnormal differentiation of keratinocytes. In summary, we describe a novel pathway for the anti-inflammatory and anti-proliferative effects of retinoids. This novel mode of retinoid action involves the interdiction of NF-IL6 signal transduction pathway by RARs in a ligand-dependent manner. This pathway can be therapeutically exploited by the systematic chemical synthesis of more potent anti-NF-IL6 retinoids. Such retinoids may exhibit increased therapeutic:toxic ratios for the treatment of certain retinoid-responsive diseases involving NF-IL6 as one of the pathophysiological signals.

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Addendum—During the review of this manuscript, two supporting papers were published describing the functional significance of RAR-NF-IL6 antagonism in the inhibition of adipogenesis and Kaposi’s sarcoma cell proliferation (25, 26).

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