Regulation of Gli Activity by All-trans Retinoic Acid in Mouse Keratinocytes

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Abstract

Sonic hedgehog (Shh) signaling is essential for many normal developmental processes. The Shh signal is interpreted by the Gli transcription factors. Elevated Gli-1 expression has been associated with several neoplasms, including basal cell carcinoma. All-trans retinoic acid (RA) has strong effects on epidermal growth and differentiation and has been used for the treatment of various epithelial disorders. In this report, we show that RA can inhibit Gli activity in immortalized murine keratinocytes in a RA receptor-specific manner. This inhibition may occur, at least in part, through sequestration of the transcriptional coactivator cyclic AMP-responsive element-binding protein-binding protein and suggests a novel effect of retinoid excess on Shh signaling.

Introduction

BCC is the most common type of skin cancer in Caucasian populations. Studies of sporadic and familial BCC have indicated that a majority of these tumors show aberrant activation of the Shh pathway (1–3). Inactivating mutations of Ptc, a putative receptor and negative regulator of Shh signaling, have been identified in familial BCCs and sporadic BCCs, whereas activating mutations in Smo, a positive regulator of the Shh pathway and the target of Ptc repressor, have also been observed in sporadic BCCs (1). These mutations lead to elevated expression of Gli-1 (and Gli-2 in some cases), which is believed to play a causative role in BCC formation. Ptc mutation is the underlying basis of basal cell nevus syndrome, which is also associated with familial BCC as well as medulloblastoma and meningioma (4). These tumors are also seen in Ptc null mutant mice, and both tumor types exhibit elevated expression of Gli-1 (5). Overexpression of Gli-1 in frog skin causes tumors (2), and its induction in murine epidermis (via a Shh transgene) results in BCC (3). The possibility that Gli family members could serve as proto-oncogenes is also consistent with discovery of Gli-1 amplification in human glioma and sarcoma (6, 7).

Vitamin A derivatives (retinoids) play central roles in embryonic development and in the maintenance of various tissues in the adult (8, 9). Retinoids also exhibit potent antitumorigenic properties in many model systems and show potential for the treatment of a number of human malignancies, including diverse epithelial cancers or precancerous lesions (10–12).

The retinoid signal is transduced by two families of nuclear receptors, the RARs (RARα, RARβ, RARγ, and their isoforms) and the RXRs [RXRα, RXRβ, RXRγ, and their isoforms (13)]. RARs function as ligand-inducible transcription regulators by binding, together with a RXR partner, to cis-acting RA response elements. RARs can be activated by both RA and its stereoisomer, 9-cis RA, whereas RXRs are activated only by 9-cis RA. RXRs are also heterodimeric partners for other nuclear receptors, including thyroid hormone, vitamin D, peroxisome proliferator activated receptor, and several orphan receptors.

Normal epidermis expresses RXRγ and RARα as well as RXRα and RXRβ, with RARγ and RXRα believed to be the predominant heterodimer (14). Although RAR target genes in skin are largely unknown, retinoid signaling is essential for epidermal development and maintenance. In addition to this, epidemiological studies and clinical trials also suggest that retinoids may reduce the incidence of non-melanoma skin cancer (15).

RARs also function in a ligand-dependent manner to inhibit AP-1 activity, and it has been suggested that the antitumorigenic effects of retinoids may occur through this mechanism (16). The basis for this trans-repression is believed to be due in part to competition for limited amounts of ancillary factors, such as CBP or p300, which are common to both AP-1 and RAR transcription complexes (17). Because both the Drosophila homologue of the Gli transcription factor family, cubitus interruptus, and vertebrate Gli-3 have been shown to use CBP as a coactivator (18, 19), Gli signaling is a potential target for RA-induced trans-repression. To test this hypothesis, we investigated the effects of RA treatment and RAR expression on Gli signaling activity in transformed mouse keratinocytes. These cell lines express Gli-2 and Gli-3, whereas Gli-1 is not detectable. We found that RA excess inhibited the activity of a Gli reporter and that this inhibition could be partially reversed by exogenous CBP or p300. Attenuation of Gli activity also paralleled Gli-2 levels, and overexpression of Gli-2 partially restored activity. Inhibition was not observed in RARα null keratinocytes and was minimal in RARγ mutant cells, demonstrating that this effect was mediated principally via RARγ.

Materials and Methods

Cell Culture and Reagents. The RAR null keratinocyte cell lines were established from primary keratinocytes by immortalization with dominant negative p53 as described previously (20). The cells were grown in SMEM supplemented with 10% chexel-treated FCS, (the calcium concentration was adjusted to 0.5 mM after treatment), 5 μg/ml insulin, 0.5 μM hydrocortisone, 1.5 mM MgCl2, 1.2 × 10−5 M cholera toxin, 24 μg/ml adenine, 10 ng/ml epidermal growth factor, and 10 μg/ml gentamycin at 37°C in 5% CO2 in air in a humidified atmosphere.

Transient Transfection and Gli Reporter Activity Assay. Mouse cDNAs for Gli-1, Gli-2, Gli-3, and Ptc, Smo, and suppressor of fused were obtained by reverse transcription of RNA from newborn mouse skin followed by PCR. Expression plasmids for human Gli-1, Gli-2, and Gli-3 as well as the Gli-responsive element reporter plasmid were provided by Dr. C. C. Hui (The Hospital for Sick Children, Toronto, Canada). The p300 and CBP expression plasmids were obtained from Dr. T. H. Snyder (Stanford University, Stanford, CA).

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4 The abbreviations used are: BCC, basal cell carcinoma; RA, all-trans retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; CBP, cyclic AMP-responsive element-binding protein-binding protein; Shh, sonic hedgehog; Ptc, patched; Smo, smoothened; AP-1, activator protein 1; SMEM, 5-minimal essential medium.
of RA on the Gli reporter. This could be due to events specific to RARγ or to the prevalence of this receptor type (relative to RARα) in these cell lines (20). To address this issue, the RARγ−/− line (which is devoid of all RARs; Ref. 20) was transfected with the Gli reporter and various amounts of RARα or RARγ expression vectors, and reporter activity was assessed in the absence or presence of RA. Both RARα and RARγ isoforms inhibited reporter activity to similar degrees in a dose-dependent manner (Fig. 1B). This suggests that the differential effects of RA ablation on Gli activity are due to the relative expression levels of receptor types.

Effects of RA on Expression of Constituents of the Shh Signaling Pathway. To further investigate the basis for the impact of retinoid treatment on Gli activity, we determined the effect of treatment on the expression of several components of the Shh signaling pathway in wild-type and RAR null lines. In untreated cultures, Gli-2 expression was comparable across all four cell lines, with the exception of reduced levels in the RARα null cell line (Fig. 2). RA reduced Gli-2 levels in both wild-type and RARα−/− cultures, and this effect was not observed in either RARγ−/− or RARαγ−/− cell lines. Ptc transcript abundance and RA response across the various cell lines were similar to that of Gli-2. This observation suggests a relationship between expression of these two factors, consistent with previous studies demonstrating regulation of Ptc by Gli. Gli-3 expression was similar in wild-type and RARα−/− cell lines but was elevated in RARγ−/− and RARαγ−/− cultures. Gli-3 transcripts were not affected by RA regardless of RAR status. The expression levels of Smo (Fig. 2) and suppressor of fused (data not shown) did not vary significantly among the cell lines. Gli-1 and Shh were not detected.

A Role for CBP in RA Repression of Gli. As has been shown for many other transcription factors, such as RARs, Gli-3 transcriptional activity is augmented by the coactivator CBP (18). Whether or not Gli-2 exhibits similar cofactor requirements has not yet been reported. To address the possibility that RA excess might affect Gli function by sequestration of such a common ancillary factor, we tested the effects of exogenous CBP or p300 on Gli reporter activity after RA treatment.

CBP or p300 transfection in wild-type cells resulted in a dose-dependent increase in Gli reporter activity in the absence of RA (Fig. 3A). When calculated as fold inhibition, it was found that CBP was very effective at reversing the inhibitory effects of RA, with a gain of up to 60% of activity seen in untreated cells (Fig. 3B). Interestingly, p300 was much less effective in reversing the effects of RA, suggesting that CBP may be preferred over p300 in this context. This difference is not due to lack of expression or function of p300 because...
untreated cells transfected with the p300 expression vector exhibited a dose-dependent gain of reporter activity comparable to that seen with CBP transfection (Fig. 3A).

As described above, RA also has an effect on Gli-2 expression, and this could contribute to the observed inhibition of Gli transcriptional activity. To assess this, vectors encoding the various Gli members were cotransfected with the Gli reporter, and activity was assessed in the absence or presence of RA. Overexpression of Gli-2 resulted in a large (7–8-fold) increase in basal Gli activity, whereas Gli-1 and Gli-3 were less potent (Fig. 3A). This suggests that Gli-2 may be the main mediator of Gli activity in this system, an observation that is further supported by our inability to detect Gli-1 and by the low levels of Gli-3 in wild-type cultures. However, all three Gli members were very potent at reversing the inhibitory effect of RA on Gli activity, with a recovery of 70–80% of expression relative to the untreated control (Fig. 3B).

Although the rescue effect was very pronounced with CBP and with different Gli members, repression of Gli activity was never completely abolished. This observation suggests that both titration of limiting cofactors and inhibition of Gli family member expression by RA may act in concert to attenuate Gli activity in these transfectants.

Discussion

The RARs can trans-repress the activity of certain transcription factors, such as AP-1, through a mechanism implicating titration of mutual coactivators such as p300 and CBP (17). This mechanism has been described in a number of systems and is thought to underlie at least some of the pharmacological effects of retinoids on tumor growth (16). Interestingly, evidence suggests that similar trans-repression activity is intrinsic to the normal function of the glucocorticoid receptor (21), suggesting that such interactions may also be physiologically relevant with regard to the RARs.

The interaction between AP-1 and RARs predicts that other transcription factors that use p300/CBP, such as Gli-3, should be affected in a similar manner. Consistent with this, we found that wild-type and RARα null cell lines exhibited a profound attenuation of Gli activity by RA. In marked contrast, the RARγ null cell line showed minimal effects, and RARαγ null cells were not responsive. Whereas reconstitution experiments suggest that this effect can be mediated equally by RARα or RARγ, there appears to be insufficient RARα to elicit an outcome in RARγ null cultures. These results correlate well with our previously reported effects of RA on AP-1 (20) and suggest that a common basis underlies the inhibitory effect of retinoid treatment on Gli and AP-1 signaling.

Multiple mechanisms have been shown to regulate Gli activity. These include modulation of expression, availability of coactivators, protein kinase A activity, proteolytic processing, and subcellular trafficking (22). It is possible that any or all of these events could be affected by RA treatment. We investigated two of these, coactivator segregation and gene expression, to attempt to explain the effect of RA on Gli activity.

Transfection of either CBP or p300 induced basal Gli activity in wild-type keratinocytes to similar degrees, suggesting that, as in other systems, CBP and p300 are functionally similar with regard to transcription through a Gli-responsive promoter. In contrast, CBP was more efficient in attenuating the inhibitory effects of RA. The reversal by CBP is not surprising, given that both Gli-3 and cubitus interruptus, the Drosophila homologue of Gli, also use CBP as a coactivator (18, 19). However, the relative ineffectiveness of p300 in evoking a similar response may be indicative of differential interaction with the RARs. Differential effects manifested by these two highly homologous proteins have been suggested previously for RA-induced differentiation of F9 embryocarcinoma cells (23).

Northern blot analysis demonstrated that RA reduced Ptc and Gli-2 levels. The effects of transfection studies indicate that Gli-2 is a potent activator of the reporter, hence, this attenuation of Gli-2 expression may contribute to RA-induced repression of the Gli pathway. Although Gli-1 and Gli-3 were not as potent at affecting basal expression levels of the reporter, all three were equipotent at reversing RA repression. However, it is unclear whether all three mediated this effect via a common mechanism. Indeed, to date, only Gli-3 has been shown to functionally associate with CBP.

Given that Gli-2 and Ptc are known targets of Shh signaling and are thus regulated by Gli, it is possible that the down-regulation of Gli-2 and Ptc by RA is secondary to a reduction in Gli signaling. In such a model, CBP sequestration would lead to decreased Gli activity, which in turn would result in decreased expression of Gli target genes, including Gli-2 and Ptc. The ability of CBP to reverse this effect is consistent with such a model.

Shh signaling is essential for normal skin development and proliferation. In our model, Shh and Gli-1 transcripts were undetectable, indicating that the observed basal activity is not dependent on Shh. In contrast, BCCs often overexpress Gli-1 due to inappropriate activation of the Shh pathway (24). Although there are a number of contradictory studies, BCC does not always respond positively to retinoid therapy (25). This may be due to the genetic basis of these tumors because Gli-1 does not require CBP for efficient transcriptional activity and may not be affected by RA. It remains to be seen whether the present
observations are unique to this particular model system or whether RA can affect Shh signaling in BCCs. RA has been shown to impact Gli and Shh expression in Xenopus and Shh expression in mouse embryos (26, 27). Moreover, we have found that RA treatment also alters Gli-1 expression in late gastrulation mouse embryos (our preliminary results). These observations further support our findings suggesting interplay between these important signaling pathways.

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References

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