The proteosome inhibitor MG132 attenuates Retinoic Acid Receptor trans-activation and enhances trans-repression of Nuclear Factor κB. Potential relevance to chemo-preventive interventions with retinoids

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Abstract

Background: Nuclear factor kappa B (NFκB) is a pro-malignant transcription factor with reciprocal effects on pro-metastatic and anti-metastatic gene expression. Interestingly, NFκB blockade results in the reciprocal induction of retinoic acid receptors (RARs). Given the established property of RARs as negative regulators of malignant progression, we postulated that reciprocal interactions between NFκB and RARs constitute a signaling module in metastatic gene expression and malignant progression. Using Line 1 tumor cells as a model for signal regulation of metastatic gene expression, we investigated the reciprocal interactions between NFκB and RARs in response to the pan-RAR agonist, all-trans retinoic acid (at-RA) and the pan-RAR antagonist, AGN193109.

Results: At-RA [0.1–1 μM] dose-dependently activated RAR and coordinately trans-repressed NFκB, while AGN193109 [1–10 μM] dose-dependently antagonized the effects of at-RA. At-RA and AGN193109 reciprocally regulate pro-metastatic matrix metalloprotease 9 (MMP 9) and its endogenous inhibitor, the tissue inhibitor of metalloprotease 1 (TIMP 1), in a manner consistent with the putative roles of NFκB and RAR in malignant progression. Activation of RAR concurs with its ubiquitination and proteosomal degradation. Accordingly, the proteosome inhibitor, MG132 [5 μM], blocked RAR degradation, quelled RAR trans-activation and enhanced RAR trans-repression of NFκB.

Conclusion: We conclude that reciprocal interactions between NFκB and RARs constitute a signaling module in metastatic gene expression and malignant progression and propose that the dissociative effect of proteosome inhibitors could be harnessed towards enhancing the anticancer activity of retinoids.

Background

NFκB (p50/p65 heterodimer) is a ubiquitous transcription factor that binds to promoter sequences (κB sites), to modulate the expression of a wide array of genes implicated in diverse cellular processes. NFκB activity is primarily regulated by cytosolic retention through interactions
with IkBα that mask its nuclear localization sequence. Activation (nuclear translocation) of NFκB proceeds through activation of the serine-specific multi-component IkB kinase (IKK), which phosphorylates IkBα at two conserved N-terminal serine residues and signals for the ubiquitination and proteosomal degradation of IkBα [1,2]. Oncogenic kinases [3,4] and physico-chemical stressors such as the hypoxic conditions and pro-inflammatory content of the tumor microenvironment [5,6] contribute to the hyperactivated state of NFκB in cancer, and its fundamental implications in cellular de-differentiation and proliferation [7,8], the subversion of apoptosis [8-10], the induction of neo-angiogenesis, invasive growth and metastasis [11-13].

Using a genetically engineered IkBα with critical serine substitutions that hinder signal-induced degradation, we [9], and others [12,13] have demonstrated that suppression of NFκB activity decreases malignant progression. Interestingly, NFκB reciprocally regulates putative pro-metastatic and anti-metastatic factors [9]. While the induction of pro-metastatic gene expression is consistent with the transcription activating function of NFκB, anti-metastatic gene repression is a mechanistic caveat. Through microarray profiling and differential gene expression analyses of a murine lung alveolar carcinoma cell line (WT-Line1) and its non-malignant counterpart transduced with a dominant negative inhibitor of NFκB (mIkB-Line1), we identified the reciprocal induction of retinoic acid receptors (RARs). Based on the mutually antagonistic interactions between NFκB (p65) and multiple members of nuclear receptor superfamily [14,15], and given the auto-inductive property of nuclear receptors [16], we postulated that dominant negative inhibition of NFκB allowed for RAR signaling and the induction RAR and anti-metastatic gene expression.

Conversely, RAR ligands, the retinoids, have established anticancer properties [17-19], although clinical use is limited by drug toxicity that is ascribed to non-specific gene trans-activation [20,21]. Mechanistically, RARs in obligate heterodimeric partnership with retinoid X receptors (RXRs), bind to gene regulatory sequences (retinoic acid response elements) where they function as transcriptional switches (“on-off”) in response to ligand receptor occupancy (“agonist-antagonist”) [22,23]. In the “off” state, receptors recruit transcriptional co-repressors with intrinsic histone deacetylase activity to the DNA template. The functional result is the deacetylation of core histones, chromatin condensation and active gene repression. The “on” state is initiated by agonist binding and proceeds through structural receptor trans-conformations that dislodge co-repressors and recruit co-activators with intrinsic histone acetylase activity. The functional result is the acetylation of core histones and chromatin relaxation, which permits the assembly of a multi-protein transcription initiating apparatus, the enhanceosome [24]. As an inbuilt resetting mechanism and to accommodate for transcription elongation, RAR trans-activation concurrently with its sequential phosphorylation, ubiquitination and proteosomal degradation [25,26].

Repression of NFκB by ligand activated RARs has not been formally explored as a putative mechanism for the anticancer properties of retinoids. Furthermore, the distinct role that proteosome degradation plays in NFκB (activation) and RAR (repression) signaling schemes is compelling as a strategy for limiting retinoid toxicity while potentiating its anticancer activity. Using WT-Line1 and mIkB-Line1 cells as models for signal regulation of metastatic gene expression, we investigate the ligand dependent interactions between NFκB and RARs and explore the potential role of proteosome inhibitors in enhancing NFκB antagonism while moderating RAR gene trans-activation and possibly retinoid toxicity.

**Results**

**Reciprocal induction of Retinoic Acid Receptors (RARs) by NFκB blockade**

Contrasting RAR transcript levels in WT and mIkB-Line 1 tumor cells by RT-PCR, we demonstrate the induction of all RAR subtypes in mIkB-Line 1 tumor cells (Fig 1A). Although all RAR subtype transcripts are detected, only RARβ protein is detectable and demonstrably enhanced in mIkB-Line 1 tumor cells (Fig 1B). Accordingly, basal RAR reporter activity is five fold induced in mIkB-Line 1 tumor cells, relative to their WT counterparts (Fig 1C).

**Ligand modulation of RAR trans-activity reciprocates NFκB trans-activity**

In WT-Line1 cells, the pan-RAR agonist, at-RA dose dependently activates RAR trans-activation. Using 1 µM at-RA as the optimal dose for induction of RAR reporter activity, co-incubation with the pan-RAR antagonist AGN193109 (1–10 µM), results in a dose-dependent decrease in RAR reporter activity (Fig 2A). Consistent with the inverse antagonistic property of AGN193109 [27], 1–10 µM concentrations of AGN193109 alone suppress RAR reporter activity below basal levels (data not shown). However, in the presence of 1 µM at-RA, 10 µM AGN193109 has an agonistic tendency.

The dose-dependent repression of NFκB reporter activity by at-RA and its reversal by AGN193109 (Fig 2B) again verifies the mutually antagonistic interactions between RAR and NFκB. In the presence of 1 µM at-RA, 10 µM AGN193109 is again observed to have an agonistic tendency. To appreciate the basis for these reciprocal signaling schemes, we assessed for RAR and NFκB (p65) interactions on artificial promoter-enhancer elements.
Suppression of NFκB signaling activity allows for the induction of retinoic acid receptor (RAR) message and protein levels and an increase in RAR signaling activity. WT-Line 1 tumor cells and their non-malignant counterparts (mIκB-Line 1) transduced with a dominant negative inhibitor of NFκB were assessed for the expression of RAR transcripts by RT-PCR, using primers specific for RAR subtypes. Exponential amplification of β-actin was utilized as a loading control (A). Western blot analysis for RAR expression in WT and mIκB-Line 1 tumor cells, using a pan-RAR antibody, demonstrates increased RAR protein levels in mIκB-Line 1 tumor cells (B) and correspondingly increased RAR reporter activity (C).
Figure 2
Ligand modulation of RAR activity with the pan-RAR agonist, at-RA and the pan-RAR antagonist AGN193109, induces reciprocal effects on NFκB signaling activity. WT-Line 1 tumor cells, transiently transfected with RAR or NFκB reporter constructs were exposed to at-RA +/- AGN193109 for 24 h at the indicated concentrations. The dose dependent induction of RAR reporter activity (A) and reciprocal repression of NFκB reporter activity (B) by at-RA is reversed by AGN193109. The results represent the average (+/- SEM) of 3 independent experiments. A consistent observation is the agonistic tendency of 10 μM AGN193109, in both RAR and NFκB reporter assays.
Activation (nuclear translocation) of NFκB precludes RAR-DNA binding activity while RAR reversibly interacts with NFκB-DNA complexes in a ligand dependent manner

WT-Line1 cells, with high basal NFκB-DNA binding activity, demonstrate considerably lower RAR-DNA binding activity in contrast to mIkB-Line1 tumor cells, with lower basal NFκB-DNA binding activity [9]. Activation of NFκB with phorbol myristate acetate [9] results in a decrease in RAR-DNA binding activity in both WT and mIkB-Line1 cells (Fig 3A). These data suggest that NFκB activation (nuclear translocation) precludes RAR-DNA binding. Identical experiments performed in the presence of an RXR DR-1 type oligonucleotide sequence demonstrate no appreciable change in RXR-DNA binding (Fig 3A), although an increase in the expression of RXR subtypes was documented in the mIkB-Line1 cells (data not shown). Furthermore, there is no change in RXR-DNA binding, following induction of NFκB translocation by PMA. Given that RXR is a heterodimeric partner for multiple members of the nuclear hormone receptor superfamily [22], it is conceivable that this assay system is overwhelmed by mass effect.

In our hands, at-RA did not affect NFκB-DNA binding in standard gelshift assays. We note however that ligand activated RXR has been reported to preclude NFκB-DNA binding activity in a cell free system that implicates higher ligand-receptor ratios than otherwise achievable [15]. To overcome the limitations of the standard gelshift assay, we utilized a gelshift oligonucleotide pull-down assay (see methods) that allows for the assessment of native protein-DNA interactions at a concentration three orders of magnitude higher than a standard gelshift assay. Using this technique, we demonstrate a dose dependent increase in RAR binding to NFκB-DNA complexes in response to at-RA (Fig 3B), and its reversal by increasing concentrations of AGN193109 (Fig 3B). These results taken together with the reporter experiments, suggest that in the “on” state (the holo receptor conformation), RARs bind to NFκB-DNA complexes and trans-repress NFκB activity while the “off” state (apo receptor conformation) is non-associative, and allows for NFκB trans-activity.

RAR reciprocally regulates pro-metastatic matrix metalloprotease 9 (MMP 9) and the anti-metastatic tissue inhibitor of metalloprotease 1 (TIMP 1)

NFκB reciprocally regulates putative pro-metastatic and anti-metastatic factors [9]. To assess the regulation of pro-metastatic and anti-metastatic gene expression by at-RA and AGN193109, we examined for changes in pro-metastatic MMP 9 and anti-metastatic TIMP 1 gene expression. We demonstrate a dose-dependent induction of TIMP 1 and reciprocal repression of MMP 9 gene expression in response to increasing concentrations of at-RA. The effects of at-RA are again mitigated by AGN193109 (Fig 4). We have previously shown, in the same model system, that the net result of the reciprocal regulation of MMP 9 and TIMP 1 gene expression by at-RA is the suppression of MMP 9 activity, tumor cell invasiveness in vitro and spontaneous metastasis in vivo [28]. We however exercise caution in speculating on the in vivo effects of AGN193109, given low doses are antagonistic while higher doses have agonistic tendency albeit not intrinsic. A fortiori, high doses of retinoid antagonists have antiproliferative (“agonist-like”) activities [29]. This underscores the notion of ligand-receptor interactions as “rheostatic” (modulators) and not “static” (agonist/on – antagonist/offset) switches.

The Proteosome inhibitor MG132 dissociates Retinoic Acid Receptor trans-activation from trans-repression of NFκB

In keeping with the rheostatic nature of RAR signaling function, at-RA dose-dependently activates RAR signaling while inducing a dose-dependent decrease in RAR protein expression. This pattern is reversed by co-incubation with AGN193109 (Fig 5A). Ligand induced ubiquitination and proteosomal degradation of RARs [25,26], is a permissive event and resetting mechanism for RAR trans-activation. Accordingly, RAR trans-activity and degradation is blocked by the proteosome inhibitor MG132 (5 μM) (5B). Although proteosome inhibitors independently inhibit NFκB activity by maintaining inhibitory IkBα protein levels, trans-repression of NFκB by ligand activated RAR is enhanced by MG132, potentially by maintaining RAR protein expression (Fig 5B). The significance of the latter mechanism is asserted by the compound repression of NFκB activity by MG132, on mIkB-Line1 cells expressing a genetically engineered IkBα, not susceptible to proteosomal degradation (Fig 5C).

Discussion

Cellular transformation and malignant progression result from an imbalance in critical positive and negative growth regulatory signals, as well as cellular factors that maintain tissue homeostasis [33]. A fundamental dynamic interplay between the mitogenic transcription factor complex, AP-1, and nuclear receptors, the arbiters of cellular differentiation, has for long been recognized and characterized as a pivotal module in the homeostatic control of cellular phenotype [34,35]. With remarkable fidelity to this model, we demonstrate a fundamental interplay between NFκB and RARs, by a mechanism that involves cross-coupling (mutually antagonistic interactions) off and on gene promoter-enhancer elements. Furthermore, we demonstrate the resulting imbalance in the expression of an extracellular protease, MMP 9 and its endogenous inhibitor TIMP 1.

Our data supports the comprehensive model that hyperactivation of NFκB in cancer results in the hyper-repression of RARs. This is consistent with the progressive
Activation (nuclear translocation) of NFκB precludes RAR-DNA interactions while RAR reversibly interacts with NFκB-DNA complexes in a ligand dependent manner. (A) RAR and RXR gelshift oligonucleotides were employed to contrast RAR and RXR-DNA binding activity in WT and mlκB-Line 1 tumor cells under basal and PMA (NFκB) induced conditions. Under basal conditions, we observe increased RAR-DNA binding activity in mlκB cells contrasted to their WT counterparts (1, 3), and a decrease in RAR-DNA binding activity upon activation of NFκB with PMA in both cell types (2, 4). No appreciable differences in RXR-DNA binding activity were observed under all experimental conditions. (B) NFκB gelshift oligonucleotides conjugated to agarose beads were used to pull down NFκB and NFκB associated proteins, from the total protein lysate of cells exposed to at-RA +/- AGN193109 for 24-h. We consistently pulled down comparable amounts of RelA-NFκB and observed a dose dependent increase in the association of RAR with NFκB-DNA complexes with increasing concentrations of at-RA, and its reversal by increasing concentration of AGN193109.
RAR reciprocally regulates anti-metastatic TIMP 1 and pro-metastatic MMP 9 in a ligand dependent and reversible manner. MMP 9 and TIMP 1 gene expression in WT-Line 1 tumor cells exposed to at-RA +/- AGN193109 for 24 hours at the indicated concentrations was assessed by real time PCR. The results represent the average of 3 independent experiments, normalized to β-actin loading controls.
decrease in RAR expression in animal models of carcinogenesis and human clinical cancer specimens [36]. Conversely, ligand activation of RAR mitigates malignant progression by repressing NFκB. Illustrative of the rheostatic nature of RAR signaling function, low doses of the pan-RAR inverse antagonist, AGN193109, de-repress NFκB activity, while higher doses are relatively agonistic in the presence of at-RA. We propose that this agonistic tendency results from decreasing the threshold for RAR activation by maintaining RAR protein levels. On the other hand, at-RA coordinately activates and induces the ubiquitination and proteosomal degradation of RAR [25,26]. These events are preceded by the sequential phosphorylation of RARs by proline-dependent protein kinases, notably cyclin dependent kinases (CDKs) and mitogen activated proteins kinases (MAPks) [37]. The latter suggests an inbuilt mechanism for the integration of mitogenic and differentiation-inducing signals in the homeostatic control of cellular phenotype.

At the system level understanding of cancer biology, "overly" simplistic models are confounded by redundancies, feed-back loops and multiple signal integration, characteristic of robust regulatory systems. With better understanding of molecular circuits and signaling schemes, we are better skilled at manipulating biological systems to desired ends. Case in point, we demonstrate that the proteosome inhibitor MG132 blocks RAR trans-activation a potentiates RAR trans-repression of NFκB. The proteosome inhibitor, MG132, quells RAR trans-activation a potentiates RAR trans-repression of NFκB. (A) at-RA induces a dose dependent decrease in RAR protein levels while increasing concentrations of AGN193109 have a restorative effect. The expression of RelA and β-actin is however not affected by at-RA +/- AGN193109. (B) at-RA (1 µM) induced degradation of RAR is blocked by the proteosome inhibitor MG132 (5 µM). Correspondingly, induction of RAR reporter activity by at-RA (1 µM) is quelled by MG132 (5 µM). (C) Basal NFκB reporter activity in mlkB-Line 1 cells is further repressed by MG132 (5 µM) by mechanism independent of the IκB-NFκB signaling axis (* P value < 0.05).

Figure 5

The proteosome inhibitor, MG132, quells RAR trans-activation a potentiates RAR trans-repression of NFκB.
degradation and quells RAR trans-activity while enhancing RAR trans-repression of NFκB (Fig 6). This repression is independent of the effect of proteosome inhibitors on the IκB-NFκB signaling cascade, given the compounded suppression NFκB responsiveness in mIκB-Line 1 cells expressing a dominant negative IκBα, not susceptible to proteosomal degradation. These results resound the promise of proteosome inhibitors in the anticancer arsenal [38-40]. We propose the combinatorial use of proteosome inhibitors and retinoids, as a strategy for enhancing chemo-preventive activity and possibly limiting retinoid toxicity.

Conclusions
Identifying NFκB as a target in the anticancer activity of retinoids provides a critical endpoint in chemo-preventive interventions. This validation yields an essential template for the assessment of intermediate endpoints of chemopreventive interventions, by establishing discernable biochemical and metabolic differences between malignant cell lines and their non-malignant counterparts with diminished NFκB activity.

Methods
The pan-RAR agonist all-trans retinoic acid (Sigma) was dissolved in 70% ethanol, to obtain a 1 mM stock solution, while the pan-RAR antagonist AGN193109 (Allergen pharmaceuticals) and MG132 (Calbiochem)
were reconstituted in DMSO to obtain a 10 mM stock solution.

**Cell lines and cell culture**

Wild type Line 1 tumor cells (WT) and their non-malignant counterparts (mLkB), transduced with a dominant negative inhibitor of NFκB were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, and maintained at 37°C in a 95% humid atmosphere, with 5% CO2.

**Expression profiling of retinoic acid receptors (RARs)**

RT-PCR was used to assess the expression levels of retinoid receptor subtypes in WT and mLkB-Line1 tumor cells. 1 µg of total RNA obtained from cell lines was reversed transcribed and amplified for RAR subtypes using the Advantage rapid RT-PCR kit (Promega®), under the following conditions: reverse transcription at 48°C for 60 min, initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturing at 95°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min; followed by a final extension of 72°C for 7 min, in a Stratagene RoboCycler™ Gradient 96 thermal cycler (Stratagene, La Jolla, CA) RAR subtype specific primers used were – RARα (5-ATGTAAGGGCCTTCITCCG-3 & 3-AGCTCTAAATGATGACCT-5), RARβ(5-CTGGCCTTGTCTCATAATTCA-3 & 3-GGTACITCCTGTCTCAGATGGAT-5), RARγ (5-ATGTAAGGGCTTCTTCCG-3 & 3-AGTCTTAATGATACTGGT-5), The expression levels of β-actin (5-GAGCTATGAGCT-3 & 3-GACAGGGATGAACACAGG-5), and RAR or NFκB were assessed under identical conditions.

**Assessment of differential DNA binding and transcriptional activity of RARs in WT and mLkB-Line 1 tumor cells**

Electromobility shift assay was used to contrast RAR-DNA binding activity of RARs in WT and mLkB following the 24 h drug exposure. The expression of RAR and p-65 NFκB were equally analyzed by western blot, for changes in the expression level of RAR and p-65 NFκB following the 24 h drug exposure.

**Physical association of RARs to NFκB-DNA complexes and the ligand responsiveness of these interactions**

WT-Line1 tumor cells were exposed to increasing concentrations of at-RA (0.1–1 µM) or increasing concentrations of AGN193109 (1–10 µM) in the presence of 1 µM at-RA for 24 h. Cells thus treated were re-suspended in 1 ml of ice cold RIPA buffer (50 mM HEPES pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.5% NP40, 1 µM PMSF and 1 µM DTT), incubated on ice for 30 min and resulting suspension pelleted by centrifugation at 14000 rpm for 10 minutes. 200 µls of the supernatant thus obtained was added to 100 µls of NFκB-oligonucleotide agarose conjugate slurry (Santa Cruz), plus 300 µls of binding buffer (10 mM Tris, pH 7.5; 50 mM NaCl; 1 mM EDTA; 5% glycerol; 1 µg/ml poly dI-dC), and incubated overnight at 4°C with constant rocking. After the overnight incubation, agarose beads were washed thrice in binding buffer, re-suspended in 30 µl of protein loading dye, boiled for 5 min and analyzed by western blot analysis. RAR or NFκB–RelA antibodies (Santa Cruz) were used in conjunction with protein A-peroxidase conjugate and immunoreactive bands were detected using the enhanced chemiluminescence system (Amersham) after exposure to Hyperfilm ECL (Amersham). 5 µg of cell lysates were equally analyzed by western blot, for changes in the expression level of RAR and p-65 NFκB following the 24 h drug exposure.

**Analysis of pro-metastastic MMP 9 and anti-metastatic TIMP 1 gene expression in response to at-RA and AGN193109 by real-time PCR**

Total RNA was extracted from control and drug exposed cells using Quaigen RNAeasy miniprep columns following the manufacturers recommendations. Total RNA thus obtained was quantified by UV absorption at 260/280 λ (Genequiant), and subjected to Northern blot analysis for the expression of MMP9 and TIMP1 as previously described. To enhance sensitivity, we utilized real time PCR analysis to appreciate changes in RAR, RelA, MMP9 and TIMP1 message levels. Briefly, 1 µg of RNA was reversed transcribed and diluted 5 fold in RNase free water. 2 µl of the cDNA thus obtained was PCR amplified in a mix of 18 µl PCR supermix (GibcoBrl) plus the 2 µl of fluorescent DNA intercalating dye SYBR green (1:3000) construct (trimerized retinoic acid receptor-beta 2 response element, generously provided by Dr M.T Underhill) or pNFκB-firefly luciferase construct (Promega) plus 2 ng of pRL-SV40 (Promega) renilla luciferase to normalize and control for tranfection efficiencies. Plasmids were incubated with 3 µl of Lipofectamine 2000 (Gibco) in serum free DMEM for 15 min, and the complex added to 70% confluent well of a 6 well plate. Experiments were performed in triplicates, and the transfection reagents scaled up accordingly.
using the real time PCR machine (Rotor-Gene 2000 Robo-cycler, Phenix research).

PCR primer pairs for MMP-9 were: 5’-TGAAGCCAAGACCAGACTCTC-3’ and 5’-TGG ACC ATA ACG CAG ACA AGA CC-3’ and for TIMP-1 were: 5’-ATG CCC ACA AGT CCC AGA AC-3’ and 5’-TACGCCAGGGAACCAAGAAG-3’ and the PCR conditions were: Initial denaturing at 95°C for 2 min, followed by 40 cycles of 95°C denaturing for 45 s, 60°C annealing for 1 min and 72°C extension for 1 min.

Statistical analyses
Experiments were performed in triplicates and results are expressed as a standard error of the mean. Statistical analyses were done using the student t-test and ANOVA.

Authors’ contributions
VBA was responsible for the study design, laboratory studies and manuscript preparation. RNR initiated these studies and provided guidance and mentorship. Both authors read and approved the final version of the manuscript.

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