c-Myc Is a Major Mediator of the Synergistic Growth Inhibitory Effects of Retinoic Acid and Interferon in Breast Cancer Cells*

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The molecular signaling events involved in the inhibition of breast cancer cell growth by retinoic acid and interferon-α were investigated. All-trans-retinoic acid and interferon-α acted synergistically to inhibit growth of both the estrogen receptor-positive breast cancer cell line MCF-7 and the estrogen receptor-negative line BT-20. In MCF-7 cells, all-trans-retinoic acid potentiated the effects of interferon-α by up-regulating the expression of the RNA-dependent protein kinase (PKR). Consequently, the synergism between all-trans-retinoic acid and interferon-α down-regulated the expression of c-Myc, but not its functional partner, Max. Transfection of MCF-7 cells with a dominant-negative mutant of PKR relieved c-Myc down-regulation and cell growth inhibition, indicating that PKR is directly involved in c-Myc down-regulation and that c-Myc down-regulation is responsible for the inhibition of cell growth. Corresponding with c-Myc down-regulation, c-Myc-Max heterodimers bound to their consensus DNA sequence were undetectable in cells treated with all-trans-retinoic acid and interferon-α, indicating diminished c-Myc functionality. When c-Myc was overexpressed ectopically via a c-Myc expression vector, MCF-7 cells became resistant to growth inhibition by all-trans-retinoic acid plus interferon-α. These experiments define the following pathway as a major pathway in the synergistic growth inhibition of MCF-7 cells by all-trans-retinoic acid plus interferon-α: all-trans-retinoic acid + interferon-α → ↑ double-stranded RNA-dependent protein kinase → ↓ c-Myc → cell growth inhibition.

Retinoids and interferons (IFNs) have each been actively investigated as anticancer agents. Although neither agent alone is effective against solid tumors, clinically encouraging results have been obtained using a combination of retinoic acid (RA) and IFNs as treatment against advanced squamous cell carcinoma of the skin and cervix and renal cell carcinoma (1). In vitro, retinoids plus IFNs have been shown to suppress growth in several lines of breast cancer cells (2, 3). This combination of agents also suppresses growth of breast and ovarian cancers in nude mice xenograft models (1). However, the molecular mechanisms involved in the growth inhibition by RA plus IFNs are largely unknown. To use these agents in a clinically beneficial manner, the RA and IFN signaling pathways and the cross-talk between them need to be defined.

Retinoic acid, one of the most active retinoids, inhibits proliferation and induces differentiation in many normal and neoplastic cells (4, 5). The biological effects of RA are believed to be largely mediated by nuclear retinoid receptors, the RARs (RARα, RARβ, RARγ, and their isoforms) and the RXRs (RXRa, RXRβ, and RXRγ and their isoforms). These receptors are ligand-activated transcription factors (5). Liganded RAR-RXR heterodimers or RXR-RXR homodimers bind to retinoid acid response elements and transduce the RA signal to the transcription machinery and the chromatin template to activate or repress the transcription of target genes (6).

Interferons have also been shown to play critical roles in cell growth and differentiation (7, 8). However, in contrast to RA, the biological effects of IFNs are mediated through species-specific, cell-surface receptors. After IFNs bind to their receptors, signal events lead to the activation of Janus protein-tyrosine kinases, phosphorylation of STAT (signal transducer and activator of transcription) proteins, and ultimately induction of gene transcription. One of the IFN-inducible genes, pkr, encodes for a double-stranded RNA-dependent protein kinase (PKR) that has been shown to be involved in tumor proliferation and differentiation (7, 8). Overexpression of PKR inhibits cell growth (9, 10), whereas expression of catalytically inactive PKR mutants results in malignant transformation and tumor formation in mice (9, 11).

We investigated the mechanism of action of all-trans-retinoic acid (atRA) plus IFN-α on growth inhibition of both estrogen receptor-positive (MCF-7) and estrogen receptor-negative (BT-20) breast cancer cells. We demonstrated that atRA potentiates the action of IFN-α by up-regulating the expression of PKR in these breast cancer cells. We then targeted our efforts to the expression of c-Myc and its functional partner, Max, since c-Myc plays critical roles in cell cycling and is overexpressed in ~30% of breast cancers (12). It has been postulated (13) that, in these cells, c-Myc expression is not efficiently down-regulated in response to differentiating agents, resulting in a reduced capacity of these cells to differentiate and an increased capacity to proliferate. c-Myc is also a transcription factor that forms heterodimers with Max and consequently binds to CACGTG enhancer elements and stimulates gene transcription (14). The dimerization of c-Myc with Max and the consequent binding to its enhancer element are a prerequisite for all known c-Myc transactivation activity (15). We investigated the hypothesis that atRA plus IFN-α can effectively down-regulate the expression of c-Myc and that PKR is directly involved in this action. These experiments show that the following pathway is a major effector of the synergistic action of atRA and IFN-α in MCF-7.
cells and possibly other breast cancer cells: atRA + IFN-α → ↑ PKR → ↓ c-Myc.

**EXPERIMENTAL PROCEDURES**

**Cell Growth Inhibition Assay—**MCF-7 and BT-20 cells (4 × 10^3 cells/well; American Type Culture Collection, Bethesda, MD) were cultured in phenol red-free Eagle's minimal essential medium (Sigma) supplemented with 5% charcoal-stripped calf serum (Sigma) in 96-well cell culture plates. Different amounts of atRA (0–1000 nM; Sigma) and IFN-α (0–1000 units/ml; Sigma) were added, and cells were incubated for 4–5 days; the medium was replaced every other day. Cells were washed, fixed with 10% trichloroacetic acid for 1 h, and then stained with 1% sulfonamide B for 1 h. Cells were washed again, and then 100 μl of 10 μM Tris-HCl (pH 10) was added to release the dye. The absorbance was measured at 562 nm. Cells not given atRA or IFN-α were designated controls; results from treated cells were expressed as a percentage of control. A plate seeded with the same number of cells and fixed immediately established 0% growth (16). Loss of cells during treatments was considered cell death and was expressed as negative values. Cells from < 15 passages were used for experiments. Solutions of atRA in absolute ethanol were made and used under lights that were covered with a UV-blocking film (CLHIC, Sydlin, Inc., Lancaster, PA). The integrity of atRA was routinely monitored by spectrophotometry.

**Stable Transfection—** MCF-7 cells grown in the same medium to 50% confluence were washed with serum-free growth medium. Two μg of a dominant mutant PKR expression construct that carried the dominant mutant PKR (pcDNA3.1/Zeo-PKRΔ6) from Dr. Antonis E. Koromilas, McGill University, Montreal, Quebec, Canada) or the c-Myc expression construct (pcSV2-c-Myc, from Dr. Andrew Henderson, Pennsylvania State University) was mixed with Lipofectin reagent (Life Technologies, Inc.) or the c-Myc expression construct (pcDNA3.1/Zeo-PKRΔ6, from Dr. Antonio E. Koromilas, McGill University, Montreal, Quebec, Canada) and added to cells for 5 h. Selection was initiated on the third day with 300 μg/ml Zeocin (Invitrogen, Carlsbad, CA) for PKRΔ6 expression and 400 μg/ml G418 (Life Technologies, Inc.) for c-Myc expression and continued for 21 days until drug-resistant colonies emerged. Colonies were pooled, grown further, and used in experiments.

**Western Blot Analysis—**Total cellular protein was separated on 8 or 12% sodium dodecyl sulfate-polyacrylamide gels. Transferred nitrocellulose blots were incubated with one of the following primary antibodies: mouse monoclonal antibody against human c-Myc (Calbiochem), rabbit polyclonal antibody against human Max, or rabbit polyclonal antibody against human c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA). After adding appropriate secondary antibodies, the blots were developed using an ECL kit (Amersham Pharmacia Biotech). The results were analyzed using EagleEye® and OneScan software (Stratagene, La Jolla, CA).

**Northern Blot Analysis—**Total RNA was isolated using Tri-Reagent, separated on 1.1 M formaldehyde and 1% agarose gels, and then transferred and cross-linked to GeneScreen nylon membranes (NEN Life Science Products). Hybridization was carried out using the following probes: Tα polynucleotide kinase-labeled antisense 40-mer of c-Myc mRNA (Oncogene Research Products, Cambridge, MA) and random primer-labeled ~850-base pair EcoRI fragment of human wild-type PKR cDNA. The results were analyzed with a phosphorimager (Bio-Rad).

**Nuclear Run-on Assay—**MCF-7 cells were lysed with Nonidet P-40 lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂ and 0.5% (v/v) Nonidet P-40 (Fluka Chemical Corp., Ronkonkoma, NY). Lysates were centrifuged at 5000 × g for 10 min and washed with 1 M salt buffer containing 50 mM Tris-HCl (pH 8.3), 40% (v/v) glycerol, 5 mM MgCl₂, and 0.1 mM EDTA and then loaded onto a 4% polyacrylamide gel. Transfer of the nuclear extract was analyzed with a phosphorimager.

**Electrophoretic Mobility Shift Assay—**To prepare nuclear extracts, untreated (control) and treated MCF-7 cells were collected, washed, and suspended in hypotonic buffer consisting of 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol, added to a low salt buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM MgCl₂, 1.2 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. Gentle dropwise addition of a high salt buffer (same as the low salt buffer, except that the KCl concentration was 0.2 M) then released soluble proteins from the nuclei. Following extraction, the nuclei were removed by centrifugation; the nuclear extract was dialyzed into a moderate salt solution; and any precipitated protein was removed by centrifugation. Fifteen μg of the nuclear extract was mixed with 10,000 cpm of Max-Max consensus oligonucleotides (5'-GGA AGC AGA CCA CGT GGT CTG CTT CC-3'; Santa Cruz Biotechnology) labeled with [γ-32P]ATP (ICN Pharmaceuticals, Inc.) using Tα polynucleotide kinase, 2 μg of poly(dI-dC):poly(dI-dC), and 300 ng/ml bovine serum albumin and loaded onto a 4% polyacrylamide gel. The gel was then dried and exposed to x-ray film. For confirming the nucleic acid-bound protein, mouse monoclonal antibody against human c-Myc and rabbit polyclonal antibody against human Max were used as specific antibodies, and a mouse monoclonal antibody against insulin-like growth factor II was used as a nonspecific antibody.

**RESULTS**

**atRA and IFN-α Synergistically Inhibit the Growth of MCF-7 and BT-20 Breast Cancer Cells—**To study the effects of retinoids and IFNs on the growth of breast cancer cells, we chose atRA because it is a natural ligand for both RAR and RXR, and it is used in clinical trials; we selected IFN-α because it is a strong inducer of PKR (8). atRA (0, 10, 100, or 1000 nM) was added alone or in combination with 0, 10, 100, or 1000 units/ml IFN-α to MCF-7 cell cultures (Fig. 1A) and BT-20 cell cultures (Fig. 1B). Cells were incubated for 4 days, and growth was measured by sulforhodamine staining. atRA alone had very little inhibitory effect on the growth of MCF-7 cells, although at the highest concentration (1000 nM), ~10% growth inhibition was observed (Fig. 1A). Similarly, IFN-α alone failed to inhibit the growth of MCF-7 cells, even at the highest concentration. But when added together, each combination of atRA and IFN-α strongly inhibited the growth of MCF-7 cells. At high concentrations of atRA (1000 nM) plus IFN-α (1000 units/ml), negative cell growth was observed, indicating cell death. Median effect analysis (17) demonstrated a synergistic inhibitory effect of atRA and IFN-α on cell growth. Similar synergistic effects have been reported using a combination of atRA and IFN-β (3). Our results for atRA differ from those reported in several other
studies (18–20) in that, at low concentrations, atRA had no growth inhibitory effect on MCF-7 cells and, at the highest dose, only marginal inhibition was observed. We believe that these differences may be attributed to our choice of dosage levels and culture medium. Specifically, in our experiments, we used relatively low concentrations of RA that are clinically achievable, and we used phenol red-free medium supplemented with charcoal-stripped serum. Phenol red is known to have estrogen-like activity (21); bovine serum contains unknown amounts of retinol, a precursor of RA, and possibly RA itself.

Similar experiments using BT-20 cells, an estrogen receptor-negative breast cancer cell line that has been shown to be refractory to RA treatment (22, 23), yielded similar results (Fig. 1B). atRA or IFN-α alone failed to inhibit the growth of BT-20 cells, but combinations of the two inhibited growth by >80% (Fig. 1B); at high concentrations, cell death was observed.

Impact of the Order of Treatment with atRA and IFN-α on the Growth Inhibition of MCF-7 Cells—To investigate the mechanism involved in the synergism of atRA and IFN-α, we investigated the impact of the order of their application on the growth of MCF-7 cells. MCF-7 cells were chosen for the remaining experiments. Cells were incubated with 500 nM atRA for 16 h and then washed with serum-free medium. IFN-α (0, 10, 100, or 1000 units/ml) was added. We used a lower concentration of atRA in this experiment because 1000 nM caused >10% growth inhibition of MCF-7 cells (see above; Fig. 1A). Preliminary experiments with 500 nM atRA showed that maximal potentiation of the IFN-α effect on MCF-7 cells occurred at 16 h of pretreatment (data not shown). As shown in Fig. 2, pretreatment with atRA endowed MCF-7 cells with responsiveness to IFN-α. As little as 10 units/ml IFN-α inhibited the growth of pretreated MCF-7 cells by >90%. At higher concentrations of IFN-α (1000 units/ml), negative cell growth (cell death) was observed. In contrast, pretreatment with 500 units/ml IFN-α for 16 h and subsequent treatment with atRA (Fig. 2) did not result in a parallel responsiveness of MCF-7 cells. Thus, we conclude that atRA is necessary to potentiate the IFN-α effect on MCF-7 cells, but the opposite does not occur under the conditions tested here.

Pretreatment with atRA Enables Transcriptional Induction of pkr by IFN-α in MCF-7 Cells—We were interested in PKR because it is an IFN-inducible protein kinase that is considered to be a primary mediator of the effects of IFN on cell growth regulation (6, 7). To investigate the molecular events involved in the enhancement of IFN-α action by atRA, MCF-7 cells were pretreated with 0, 10, 100, or 1000 nM atRA for 16 h. atRA was then removed, and 500 units/ml IFN-α was added for another 16 h. Cells were collected, and PKR mRNA and protein were analyzed using Northern blotting and Western blotting, respectively. Fig. 3 (lane 2) shows that no PKR mRNA or protein was detected in MCF-7 cells treated with atRA alone; surprisingly, no pkr gene expression was detected in IFN-α treated cells either (lane 3). However, when cells were pretreated with atRA, both PKR mRNA and protein were strongly induced by IFN-α in an atRA pretreatment dose-dependent fashion (lanes 4–6).

Because a previous study with neuroblastoma cells (24) and a recent study with breast cancer cells (25) showed that RA is able to regulate gene expression at the post-transcriptional level, we investigated whether atRA is able to regulate pkr gene transcription. Nuclear run-on assays were performed on nuclei isolated from MCF-7 cells that had been pretreated with 500 nM atRA for 16 h, followed by 500 units/ml IFN-α for 45 min. Fig. 4 shows that no pkr transcription occurred in nuclei isolated from control cells or from cells treated with 500 nM atRA or 500 units/ml IFN-α (strips 1–3). However, when cells were pretreated with atRA, pkr transcription was strongly induced by IFN-α (strip 4). β-Actin, a housekeeping gene, was transcribed normally under each of these conditions.

PKR-linked c-Myc Gene Down-regulation—Next we investigated which downstream genes are regulated by atRA- and IFN-α-induced PKR action. Because c-Myc is one of the most important regulators of the cell cycle (14) and is overexpressed in some breast cancers (12), we targeted our efforts to c-Myc and its functional partner, Max. MCF-7 cells were pretreated with 10, 100, or 1000 nM atRA for 16 h, followed by incubation with 500 units/ml IFN-α for 16 h (for RNA analysis) or 48 h (for protein analysis). Cells were collected, and protein and RNA were isolated and analyzed. As shown in Fig. 5, treatment with atRA alone (A, lane 2; and B, second bar) or IFN-α alone (A, lane 3; and B, third bar) did not affect c-Myc expression. However, pretreatment with atRA strongly down-regulated c-Myc expression in MCF-7 cells (A, lanes 4–6; and B, fourth through sixth bars). Pretreatment with as little as 10 nM atRA significantly decreased the expression of c-Myc (p < 0.01), whereas at higher concentrations of atRA pretreatment, >80% of c-Myc protein expression was suppressed. Interestingly, the changes occurring in c-Myc protein expression were not reflected in c-Myc mRNA under all experimental conditions (Fig. 6A). This result supports the hypothesis that PKR is directly involved in the growth suppression of MCF-7 cells because a major function of PKR is inhibition of protein synthesis (26). Nevertheless, as shown in Fig. 6B, PKR inhibition of protein synthesis was not global because Max expression under these same experimental conditions was unaffected.

To examine whether PKR is directly involved in the down-
regulation of c-Myc. MCF-7 cells were stably transfected with a dominant-negative PKR cDNA construct (PKRΔ6). PKRΔ6 was derived from PKR cDNA by deleting 6 amino acids between catalytic domains V and VI, and it has been shown to inhibit the actions of wild-type PKR (9). Transfectants were pooled after 26 days and grown further for c-Myc protein analysis. As shown in Fig. 7A, the PKRΔ6 transfection of MCF-7 cells resulted in relief of the suppression of c-Myc expression (fourth through sixth lanes). Growth inhibition experiments were also performed on these transfectants by pretreatment with 10, 100, and 1000 nM atRA, followed by 500 units/ml IFN-α. As shown in Fig. 7B, the growth of these transfectants was inhibited by only ~25% even at the highest level of atRA pretreatment.

Down-regulation of c-Myc in MCF-7 Cells Corresponds with Diminished c-Myc Functionality—To test whether the down-regulation of the c-Myc protein level by atRA plus IFN-α indeed corresponds with an alteration in c-Myc functionality, electrophoretic mobility shift assays were performed to assess the level of c-Myc Max heterodimers bound to their consensus DNA sequence. Dimerization of c-Myc with Max and the consequent binding to its enhancer element are a prerequisite for all known c-Myc transactivation activity (15). In untreated MCF-7 cells, the c-Myc Max heterodimers were abundant (Fig. 8, lane 1), but after treatment with 500 nM atRA and 500 units/ml IFN-α, c-Myc Max heterodimers were undetectable (lane 2). Mad Max heterodimers and Max homodimers were still present in treated cells, but in contrast to our expectation, their abundance was not increased. The binding specificity of c-Myc Max DNA complexes was confirmed by incubating untreated nuclear extracts with a mouse monoclonal antibody against human c-Myc (lane 4) and rabbit polyclonal antibodies against human Max (lane 5). When antibodies were added to nuclear extracts, they interfered with the formation of c-Myc Max, Mad Max and Max dimers and/or, consequently, binding to the consensus DNA sequence (lanes 4 and 5). As expected, a nonspecific antibody (a mouse monoclonal antibody against human insulin-like growth factor II) did not interfere with the dimerization and DNA binding of c-Myc, Mad, and Max proteins (lane 3). Mad Max heterodimers and Max Max homodimers were suggested based on the appearance of all dimers after using antibodies against Max and the migration of these dimers on the gel (Max is a 21–22-kDa protein, and Mad proteins have molecular masses of 25–34 kDa).

Ectopic Expression of c-Myc Endows MCF-7 Cells with Resistance to the Growth Inhibition by atRA plus IFN-α—Finally, we were interested in whether overexpressing c-Myc in MCF-7 cells could render resistance to growth inhibition induced by atRA plus IFN-α. MCF-7 cells were transfected with the c-Myc expression vector pSV2-cMyc. Positive colonies were pooled, grown further, and treated with a combination of 500 nM atRA and 500 units/ml IFN-α. The same treatments were also applied to vector-transfected and parental MCF-7 cells. Cell growth was measured by sulforhodamine staining. The results are expressed as a percentage of untreated parental MCF-7 cells. As documented in Fig. 9, although similar growth inhi-
Fig. 8. Diminished c-Myc DNA binding in MCF-7 cells treated with atRA plus IFN-α. Cells were untreated (lane 1) or treated with 500 nM atRA for 16 h and then with 500 units/ml IFN-α for 48 h (lane 2). Electrophoretic mobility shift assays were performed as described under “Experimental Procedures.” The dimers were verified by incubating untreated nuclear extracts with a mouse monoclonal antibody against human c-Myc (lane 3), a mouse monoclonal antibody against human c-Myc (lane 4), and rabbit polyclonal antibodies against human Max (lane 5). Mad:Max heterodimers and Max:Max homodimers were proposed based on the result of lane 5 and their migrations on the gel.

Fig. 9. c-Myc-transfected MCF-7 cells resist growth inhibition by the combination of atRA and IFN-α. Parental (○), vector-transfected (●), and c-Myc-transfected (▲) MCF-7 cells were treated with 500 nM atRA and 500 units/ml IFN-α for 1, 2, 3, or 4 days and then stained with sulforhodamine as described under “Experimental Procedures.” The results are expressed as a percentage of untreated parental MCF-7 cells and represent the mean ± S.D. for quadruplicate experiments.

Discussion

Although various retinoids have been shown to be highly effective as chemotherapeutic agents in some hematologic malignancies, single retinoid treatment of solid tumors is generally ineffective. Combinations of retinoids plus other antiproliferative agents have been investigated for the treatment of solid tumors, and clinical studies of retinoids plus IFNs have yielded encouraging results (1). To use these agents in a clinically beneficial manner, the molecular mechanisms involved in their action need definition. Here we demonstrated a strong synergistic growth inhibition by atRA and IFN-α in both an estrogen receptor-positive breast cancer cell line (MCF-7) and an estrogen receptor-negative breast cancer cell line (BT-20) in which neither atRA nor IFN-α alone was effective. Since the growth of MCF-7 cells was inhibited by IFN-α when cells were pretreated with atRA, but not the converse, we conclude that the signal transduction pathway for IFN-α is not functional in these cells, but could be restored by atRA treatment. Indeed, we found that, in MCF-7 cells treated with IFN-α alone, the expression of an important antiproliferative gene, pkr, was not detectable. Pretreatment with atRA restored the ability of IFN-α to induce the expression of Pkr. These results agree well with other recent studies (3, 30) that showed that RA is able to up-regulate STAT proteins and to enhance the action of IFNs. Taken together, these observations indicate that, in MCF-7 cells, the IFN-α signal is not transduced, and consequently, PKR is not expressed. After treatment with atRA, the IFN-α signal transduction pathway is operative, and PKR expression is strongly inducible.

PKR is an IFN-inducible gene product that has been implicated in the regulation of cell growth and tumorigenesis (26). PKR is a double-stranded RNA-dependent protein kinase that inhibits mRNA translation by phosphorylating the protein synthesis factor eukaryotic initiation factor 2. However, the inhibition of protein synthesis by PKR is not global. Rather, it has been proposed that PKR binds to and regulates the translation of a discrete set of mRNAs that encode proteins important in the control of normal cell growth. The activation of PKR may occur in a localized manner such that phosphorylation and inhibition of eukaryotic initiation factor 2 function lead to a discriminatory effect on the translation of these mRNAs (8). In this context, we demonstrated that c-Myc is regulated by PKR, whereas Max is not. Our experiments define c-myc as a downstream gene that is regulated by PKR in MCF-7 cells. In cell cultures in which PKR was not induced, c-Myc protein was expressed, whereas in cell cultures in which PKR was induced, c-Myc was down-regulated. The magnitude of the down-regulation corresponds to the level of PKR induction. Additionally, the induction of PKR and the decreased expression of c-Myc corresponded closely to the atRA- and IFN-α-induced growth inhibition of MCF-7 cells. To clearly establish the PKR link to c-Myc, we showed that the transfection of MCF-7 cells with a dominant-negative PKR cDNA (PKRΔ6) relieved the down-regulation of c-Myc expression and largely eliminated the growth inhibition of MCF-7 cells. Since dimerization of c-Myc with Max and the consequent DNA binding are a prerequisite for all known c-Myc functions (15), we performed electrophoretic mobility shift assays to determine the relative amount of c-Myc that bound to its consensus DNA sequence. Our experiments showed that, in untreated MCF-7 cells, c-Myc-Max heterodimers bound to their consensus DNA sequence were abundant, but in atRA- and IFN-α-treated MCF-7 cells, the level of this heterodimer was undetectable. These results suggest that treatment with atRA plus IFN-α indeed affects c-Myc functionality.

The c-Myc network is rather complicated. In addition to the formation of c-Myc-Max heterodimers and Max-Max homodimers, Max also forms heterodimers with proteins from the Mad protein family (27). It is believed that c-Myc and Mad proteins compete for the binding of Max inside the cell (15) and that Max-Max homodimers and Mad-Max heterodimers counteract the mitogenic actions of c-Myc-Max. The predominance of one particular dimer corresponds to the relative concentration of Max partners (28, 29). In this context, the observation that the Max-Max homodimers and Mad-Max heterodimers did
not significantly increase in cells treated with atRA and IFN-α was unexpected. To further support the hypothesis that c-Myc is the target of atRA plus IFN-α in growth inhibition, we overexpressed c-Myc by introducing a c-Myc expression vector into MCF-7 cells. These cells acquired resistance to growth inhibition by atRA plus IFN-α. Presumably, in c-Myc-transfected MCF-7 cells, ectopic expression of c-Myc was not affected by PKR, and hence, these cells became resistant to the experimental treatment.

Since high concentrations of atRA caused ~10% growth inhibition of MCF-7 breast cancer cells and since ameliorating the down-regulation of c-Myc did not totally relieve the growth inhibition caused by atRA and IFN-α, it is likely that additional pathways exist to signal the antiproliferative action of atRA and that additional cell growth regulators are targeted by atRA plus IFN-α. Nevertheless, our results show that the following pathway is a major one involved in the growth inhibition of MCF-7 cells by atRA and IFN-α: atRA + IFN-α → PKR → c-Myc → cell growth inhibition. In addition, our results provide further support for the importance of PKR and c-Myc as regulators of cell growth.

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REFERENCES

1. Lippman, S. M., Lotan, R., and Schleuniger, U. (1997) Int. J. Cancer 70, 481–483
2. Widschwendter, M., Daxenbichler, G., Dapunt, O., and Marth, C. (1995) Cancer Res. 55, 2135–2139
3. Kolla, V., Lindner, D. J., Xiao, W., Borden, E. C., and Kalvakolanu, D. V. (1996) J. Biol. Chem. 271, 10508–10514
4. Chambon, P. (1994) Semin. Cell Biol. 5, 115–125
5. Mangelsdorf, D. J., Umesono, K., and Evans, R. M. (1994) in The Retinoids (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) pp. 319–350, Raven Press, New York
6. Glass, C. K., Rose, D. W., and Rosenfeld, M. G. (1997) Curr. Opin. Cell Biol. 9, 222–232
7. Kalvakolanu, D. V., and Borden, E. C. (1996) Cancer Invest. 14, 25–53
8. Jaramillo, M. L., Abraham, N., and Bell, J. C. (1995) Cancer Invest. 13, 327–338
9. Koromilas, A. E., Roy, S., Barber, G. N., Katz, M. G., and Sonenberg, N. (1992) Science 257, 1685–1689
10. Lengyel, P. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5893–5895
11. Meurs, E. F., Watanabe, Y., Kaderete, S., Barber, G. N., Katz, M. G., Chong, K., Williams, B. R., and Hovanessian, A. G. (1992) J. Virol. 66, 5805–5814
12. Garcia, I., Dietrich, P. Y., Aapro, M., Vauthier, G., Vandax, L., and Engel, E. (1989) Cancer Res. 49, 6675–6679
13. Watson, P. H., Singh, R., and Hole, A. K. (1996) Curr. Top. Microbiol. Immunol. 213, 267–283
14. Lemaitre, J., Buckle, R. S., and Mechali, M. (1996) Adv. Cancer Res. 70, 95–144
15. Zornig, M., and Evan, G. I. (1996) Curr. Biol. 6, 1553–1556
16. Skahan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokoch, H., Kenney, S., and Boyd, M. B. (1990) J. Natl. Cancer Inst. 82, 1107–1112
17. Chou, T. C., and Talalay, P. (1984) Adv. Enzyme Regul. 22, 27–55
18. Chambon, P. (1994) Semin. Cell Biol. 5, 149–157
19. Wakeling, A. E. (1989) J. Steroid Biochem. 34, 183–188
20. Coromilas, A. E., Roy, S., Barber, G. N., Katz, M. G., and Sonenberg, N. (1992) Science 257, 1685–1689
21. Wakeling, A. E. (1989) J. Steroid Biochem. 34, 183–188
22. Marth, C., Mayer, I., and Daxenbichler, G. (1984) Biochem. Pharmacol. 33, 2217–2221
23. Fontana, J. A., Miranda, D., and Burrows, Mezu, A. (1990) Cancer Res. 50, 1977–1982
24. Higuchi, T., Hannigan, G. E., Malkan, D., Yeger, H., and Williams, B. R. (1991) Cancer Res. 51, 3958–3964
25. Langenfeld, J., Kiyokawa, Sekula, D., Boyle, J., and Dmitrovsky, E. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12071–12074
26. Samuel, C. E. (1997) Int. J. Hematol. 65, 227–237
27. Ayer, D. E., Kretzner, L., and Eisenman, R. N. (1993) Cell 72, 211–222
28. Henikoff, S., and Luscher, B. (1996) Adv. Cancer Res. 68, 110–183
29. Gupta, S., Seth, A., and Davis, R. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3216–3220
30. Matikainen, S., Ronni, T., Lehtonen, A., Sareneva, T., Melen, K., Nordling, S., Levy, D. E., and Jukunen, I. (1997) Cell Growth Differ. 8, 687–698