Retinoid Receptor-dependent and -independent Effects of N-(4-Hydroxyphenyl)retinamide in F9 Embryonal Carcinoma Cells

John L. Clifford, David G. Menter, Michael Wang, Reuben Lotan, and Scott M. Lippman

Abstract

Fenretinide [N-[4-hydroxyphenyl]retinamide (4HPR)] is a retinoid analogue with antitumor and chemopreventive activities. The mechanism of action of 4HPR is not fully understood, but it is hypothesized that this compound acts independently of the nuclear retinoid receptor pathway. To test this hypothesis directly, we have analyzed the activity of 4HPR on a panel of F9 embryonal carcinoma cell lines, which includes wild-type and mutant lines that lack expression of retinoic acid receptor γ, retinoid X receptor α, or both. 4HPR (10 μM) treatment resulted in a rapid induction of cell death in F9 cells, which was responsible for their near elimination by 48 h. This effect occurred in the receptor-null cell lines as well. Treatment of the wild-type cells for 4 days with 1 μM 4HPR also resulted in a primitive endodermal differentiated phenotype that is normally seen upon all-trans-retinoic acid treatment and is characterized by the up-regulation of laminin B1 and type IV collagen. This differentiation response did not occur in the receptor-null cells. Therefore, two distinct effects of 4HPR were identified in this system: a rapid induction of cell death and a slower induction of differentiation, which are likely to be receptor independent and dependent, respectively.

Introduction

Retinoids belong to a class of chemical compounds that includes active metabolites of vitamin A (retinol) as well as a diverse array of synthetic derivatives. Vitamin A is required for normal embryonic development, epithelial homeostasis, maintenance of reproductive capacity, and functioning of the visual cycle. Additionally, retinoids have been shown to modulate a wide variety of cellular processes, including proliferation, differentiation, homeostasis, malignant transformation, and apoptosis (1). Retinoids also act pharmacologically to restore regulation of differentiation and growth in certain premalignant and malignant cells in vitro and in vivo. Consequently, retinoids are under study as therapeutic and chemopreventive agents for a variety of cancers (2). Retinoids are also potent drugs for the treatment of severe cystic acne, psoriasis, and several other dermatological disorders (3). It is now believed that retinoids exert their effects primarily through nuclear receptor proteins. Retinoid receptors comprise two families of ligand-dependent, DNA-binding, transcriptional transactivators: RARs and RXRs, both members of the nuclear steroid hormone receptor superfamily (1, 4). The naturally occurring retinoids ATRA and 9C-RA both activate RARs, whereas RXRs are only activated by 9C-RA. Retinoid receptors bind DNA and activate transcription primarily as ligand-bound RAR/RXR heterodimers (1).

The F9 murine embryonic carcinoma cell line, a well-established model system for the study of retinoid-induced differentiation, is highly sensitive to a variety of retinoid effects (5). These cells resemble the pluripotent stem cells of the inner cell mass of the early mouse embryo and, depending on the culture conditions, differentiate into three distinct endodermal cell types (primitive, parietal, and visceral) upon treatment with ATRA. In addition to differentiation, retinoids induce both antiproliferative and apoptotic responses in these cells (6, 7). So that RAR and RXR function can be understood in greater detail, a panel of F9 cell lines that are null for the expression of RARα, RARγ, RXRα, and pairwise combinations of RARα-RXRα and RARγ-RXRα by homologous recombination-mediated gene targeting has been generated (7–10). The F9 cell receptor KO cell lines (F9 KO) have allowed a more precise determination of the degree of functional redundancy between RARs/RXRs and have demonstrated a differential requirement for RARs and RXRs in mediating retinoid-induced cell death (7). Another important finding from the F9 KO studies was that the loss of pairs of receptors (Aγ/Xα−/− and Aα/Xα−/− lines) resulted in a more severe impairment of retinoid response than for any of the single KOs (10, 11). Cells lacking expression of the most abundant RAR (RARγ) and the most abundant RXR (RXRα) in F9 cells resulted in the complete loss of measurable retinoid response (10, 11). In addition to ATRA and 9C-RA, there are numerous other naturally occurring and synthetic retinoids that can bind and activate retinoid receptors. Several recent reports suggest that some of these retinoids may act through receptor-independent mechanisms (12). One synthetic retinoid, 4HPR, inhibited carcinogenesis in animal models for various epithelial cancers and exhibited some activity in preneoplastic oral leukoplakia patients and in patients at risk of developing breast and ovarian cancer (13). Several clinical chemoprevention trials targeting breast, prostate, cervix, skin, lung, ovary, and bladder are ongoing (4). Like other retinoids, 4HPR can up-regulate the expression of RARβ (14) and activate transcription of retinoic acid response elements by RARs (15, 16). Also, RARβ expression was associated with the antiproliferative action of 4HPR in ovarian cancer cells (17). However, the ability of 4HPR to induce apoptosis in cells that are resistant to ATRA suggests that this activity may not involve retinoid receptors in some cell types (12, 18–20). To directly test the hypothesis that 4HPR can act through a receptor-independent mechanism, we have measured its activity on the F9 KO cell lines that are WT, RARγ null (RARγ−/−), and RXRα null (RXRα−/−) and, particularly, on the cells essentially lacking retinoid receptor function (Aγ/Xα−/−). The Aγ/Xα−/− cells also exhibit a greatly reduced up-regulation of RARβ expression upon ATRA treatment compared to WT cells (10) and should, therefore, allow deter-
mination of any strict requirement for RARβ action in the antiproliferative and/or apoptotic effects of retinoids.

Materials and Methods

Cell Lines and Cell Culture. WT F9 cells (F9 WT) and mutant clones lacking expression of RARγ (RARγ<sup>−/−</sup>), RXRα (RXRα<sup>−/−</sup>), or both RARγ and RXRα (AγXα<sup>−/−</sup>) proteins were cultured and induced to differentiate into primitive endoderm, as described previously (7, 8). ATRA and 4HPR were obtained from Sigma Chemical Co. (St. Louis, MO). Stocks were made in ethanol to a concentration of 1 mM and diluted to the appropriate final concentrations in culture medium. Cells were photographed using a phase contrast microscope at a magnification of ×200.

Cell Viability Assay. A calcein AM fluorescence-based cell viability assay (21) was performed as follows. Cells were plated in triplicate in 96-well plates and treated with 10 μM ATRA or 4HPR at varying times. To detect the relative number of viable cells versus dead cells per well, we added the fluorescent dye calcein AM to the medium (1 μM), 1 h prior to the termination of the assay. The plates were fluorometrically analyzed using a Cytofluor 2300 fluorescence plate reader (Millipore). The excitation and emission wavelengths for calcein were set at 485 nm and 530 nm, respectively. The intensity of fluorescence was measured for each well, and the results were represented as percentage inhibition of cell viability relative to untreated controls (columns). Bars, SE for triplicate cultures.

Apoptosis/Cell Death Assays. The occurrence of apoptosis and necrosis was visualized using a modified PI-Hoechst 33258 double staining assay (22). Cells were plated at a density of 4 × 10<sup>3</sup> cells per 10-cm dish containing glass coverslips coated with CellTak (Collaborative Biomedical Products) and allowed to attach overnight, before treatment with 10 μM 4HPR for 24 h. PI was added to the growth medium to a final concentration of 20 μg/ml, 1 h prior to the end of the assay. The coverslips were placed in six-well plates containing fresh growth medium containing 5 μg/ml Hoechst 33258 dye. Coverslips were incubated at 37°C for 15 min, medium was aspirated, and the slips were fixed in 1% paraformaldehyde for 30 min at room temperature. After a brief period of drying, the coverslips were mounted on slides using ProLong mounting medium (Molecular Probes, Eugene, OR). The cells remaining in the six-well plates were trypsinized and combined with the culture supernatant, followed by resuspension in growth medium containing 5 μg/ml Hoechst 33258 dye and incubation at 37°C for 15 min. The cells were then spun and resuspended in 1 ml of PBS. Approximately 3 × 10<sup>4</sup> cells were attached to slides using a Cytospin centrifuge (Shandon and Lipshaw Inc.) and observed visually for quantitation of apoptosis and necrosis. Apoptotic cells were scored as those that exhibited the DNA condensation characteristic of apoptosing cells, visible upon Hoechst 33258 staining, but did not stain intensely with PI. Necrotic cells were scored as those staining substantially above background levels with PI. The percentage of apoptotic and necrotic cells was calculated for at least 150 cells per sample, in three or more separate fields. The cells remaining on coverslips were photographed on a fluorescence microscope sequentially using filters for both Hoechst 33258 dye and PI, at a magnification of ×400.

RT-PCR. RNA preparation, RT-PCR, and Southern blotting were performed as described previously (7). The PCR primers and probes for collagen Iα1, laminin B1, and 36B4 were described previously (7).

Results

4HPR Induces a Rapid Cell Killing in the Absence of Retinoid Receptors. Several previous studies have indicated that 4HPR may exert its effects through a retinoid receptor-independent mechanism (15, 18–20). To answer this question directly, we analyzed a panel of mutant F9 embryonal carcinoma cell lines that lack RARγ, RXRα, or both proteins. These cells were treated with 10 μM 4HPR for 24 and 48 h, and cell viability was measured using the calcein AM fluorescence-based viability assay (21). All three receptor-null cell lines exhibited a reduction in cell viability similar to the parental F9 WT cell line (Fig. 1A). For comparison, the cells were also treated with 10 μM ATRA, which is a primary biologically active retinoid. At this dose, there is a minimal loss of cell viability after 48 h of treatment, in keeping with previous reports (Fig. 1B; Ref. 7–11). Longer treatment (4–5 days) with ATRA and other retinoids resulted in an induction of PrE differentiation, inhibition of cell proliferation, and induction of apoptosis in the WT but not in the receptor-null cells (data not shown; Refs. 7–11). These later retinoid effects are distinct from the rapid receptor-independent reduction in cell viability induced by 4HPR.

The Cell Growth-inhibitory Effect of 4HPR Is Due to Induction of Both Apoptosis and Necrosis. We have shown previously that the delayed apoptotic response to ATRA and other retinoids is dependent upon RXRα but not RARγ (7). To determine whether 4HPR-induced cell death involves apoptosis and/or necrosis, we conducted a dual-stain procedure with the fluorescent DNA-binding dyes Hoechst 33258 and PI, which are membrane permeable and impermeable, respectively. Cells undergoing apoptosis exhibit a distinct morphology, characterized by condensation and fragmentation of nuclear DNA, which can be readily detected by intense staining with Hoechst 33258. Treatment of both WT and AγXα<sup>−/−</sup> cells with 10 μM 4HPR resulted in the appearance of apoptosing cells (Fig. 2A, middle, arrows). In contrast to apoptosing cells, necrotic cells lose membrane integrity and leak their contents into the surroundings. Such cells are able to take up PI, which results in an intense red staining of DNA. As seen for apoptosis, both WT and AγXα<sup>−/−</sup> cells undergo necrosis upon 4HPR treatment (Fig. 2A, right, arrowheads). Thus, both apoptosis and necrosis contribute to the rapid loss of cell viability induced by 4HPR.

To determine whether either mechanism of cell killing is mediated preferentially by RARγ or RXRα, we made an attempt to quantitate apoptotic and necrotic cells by visual inspection of the Hoechst 33258-PI-stained slides. There was an ~3-fold higher percentage of apoptosing cells for the RARγ<sup>−/−</sup> cultures, compared to the other cell lines (WT, RXRα<sup>−/−</sup>, and AγXα<sup>−/−</sup>) after 24 h of treatment with 10 μM 4HPR (Fig. 2B). The higher apoptosis count for the RARγ<sup>−/−</sup>
cells could be due to clonal variation in sensitivity to 4HPR, which is unrelated to receptor status. Further analysis of multiple, independently generated RARγ−/− clones would be necessary to verify whether RARγ loss is related to a higher apoptosis rate.

**4HPR Induces Differentiation of WT but not AγXα−/− Cells.** Because there is evidence in several systems that 4HPR can act in a receptor-dependent manner (14–17), we attempted to determine whether 4HPR treatment caused any measurable effects on the WT cells that differed from effects on the receptor-null cell lines. Different 4HPR effects in WT cells would likely be due to the presence of retinoid receptors. Several cultures each of WT and receptor-null cells were maintained in the presence of 10 μM 4HPR for up to 3 weeks. A small percentage of cells of each type survived the early cell killing effects. However, only the WT cells (but not the receptor-null cells) had a differentiated morphology resembling ATRA-treated WT cells (data not shown). To better determine whether there may be a receptor-dependent induction of differentiation by 4HPR, we treated cells with 1 μM 4HPR, which does not result in appreciable apoptosis or necrosis (data not shown), to allow the observation and analysis of a sufficient number of cells. 4HPR induced a morphological differentiation of WT cells, but not AγXα−/− cells, which was similar to PrE induction by ATRA (Fig. 3A). RT-PCR was then used to determine the expression of two established markers of PrE differentiation, laminin B1 and type IV collagen, to further confirm PrE differentiation by 4HPR. Treatment of the WT cells but not the AγXα−/− cells for 4 days with either 1 μM ATRA or 1 μM 4HPR resulted in a similar induction of both markers (Fig. 3B).

**Discussion**

It has previously been shown that 4HPR can act in a manner resembling classic retinoids, by the apparent activation of the nuclear retinoid receptor pathway (14–17). This is in seeming contradiction to other findings showing that 4HPR binds poorly to the RARs and inhibits cell proliferation and/or induces apoptosis in cells that are resistant to ATRA (15, 18–20, 23). Our finding that receptor-deficient, ATRA-resistant cells are as sensitive as WT cells to the cell-killing effects of 4HPR, are in agreement with the latter study data. However, the finding that 4HPR can induce PrE differentiation in only the WT cells and not the AγXα−/− cells, i.e., likely in a receptor-dependent manner, is in agreement with the former study data. It should be noted that reexpression of RARγ in the RARγ−/− cells (24) and RXRα in the RXRα−/− cells results in complete restoration of ATRA response, including the up-regulation of differentiation markers. This indicates that the absence of ATRA-induced differentiation in the receptor-null cells is truly the result of receptor loss and that the absence of 4HPR-induced differentiation in these same cells is highly likely to also be the result of receptor loss. Therefore, 4HPR may be acting both through a receptor-dependent mechanism, for the initial cell killing events, and through the RXRs, for the latter differentiation events. Fig. 4 illustrates the potential dual function of 4HPR. The actions of this compound can be conceptually divided into two categories: receptor-mediated and receptor-independent actions. It is theoretically possible that both categories of action are interrelated, but our results demonstrate that, at least for F9 cells, the receptor-independent early events are completely unaffected by receptor status. The potentially receptor-mediated differentiating effects, which take 4–6 days to manifest, are, of course, not observable in cells killed after 1 or 2 days of treatment. It appears, therefore, that 4HPR exerts two separate, unrelated effects: early cell killing and late differentiation.

Because 4HPR is known to bind RARs poorly (19), it is not clear how it might act through the receptors to induce differentiation. One possibility is that 4HPR is displacing sufficient quantities of ATRA or other bioactive retinoids from intracellular stores to activate the receptors. Such retinoids or their precursors could originate in the FCS that is supplemented to 10% in the culture medium. The displaced retinoids would then be free to enter the nucleus and activate the retinoid receptors in the usual way. This seems unlikely, however, in light of data from other investigators who have shown that 4HPR could inhibit anchorage-independent cell growth, which is a late effect

---

**Fig. 2.** 4HPR induces both apoptosis and necrosis. A, F9 WT and AγXα−/− cells were treated with 10 μM 4HPR for 24 h and stained in situ sequentially with Hoechst 33258 (Hoechst) and PI. Identical fields were photographed with filters revealing either the blue fluorescence of Hoechst (left and middle) or both the blue Hoechst and red PI fluorescence simultaneously (right). Arrows, Hoechst 33258-stained cells containing nuclei with features characteristic of apoptosis; arrowheads, necrotic cells, which stain intensely with the membrane impermeable PI. B, both adherent and floating F9 WT and AγXα−/− cells from the corresponding culture wells shown in A, along with identically treated RARγ−/− and RXRα−/− cells, were combined for quantitation of apoptosis and necrosis. Hoechst-stained cells containing nuclei with features characteristic of apoptosis were scored as apoptotic while cells staining intensely with PI were scored as necrotic. Columns, percentages of the total numbers of staining nuclei for apoptotic and necrotic cells.
scored after 14 days of treatment, even in retinoid-depleted, charcoal-stripped serum (17). Unfortunately, this possibility cannot be tested directly with F9 cells because they do not survive in stripped serum or in serum-free medium. Another plausible explanation for the ability of 4HPR to induce differentiation may be related to its ability to alter retinoid metabolism in cells, such that either synthesis of ATRA or other retinoids is enhanced or their catabolism is inhibited. In support of this idea are reports that, in rat and human tissues, 4HPR can inhibit the activity of enzymes involved in retinol esterification such as acylcoenzyme A:retinol acyltransferase and lecithin:retinol acyltransferase (25). Inhibition of retinol esterification could make greater amounts of retinol available for conversion to ATRA. In keeping with this explanation, another study showed that 4HPR inhibited ATRA catabolism in NB4 promyelocytic leukemia cells (26). This effect coincided with an enhancement of ATRA-induced differentiation. In contradiction to these results is the observation that patients treated with 4HPR have reduced levels of plasma retinol (27). Additionally, 4HPR treatment reduced retinol levels in several organs in rats (13). These results suggest that, at least in the whole organism, alterations in retinoid metabolism by 4HPR tend to favor an overall decrease, rather than an increase in available bioactive retinoids.

A third explanation for the differentiating effect of 4HPR suggests that it is converted in some way to a retinoid, which can act through the retinoid receptors. For cells exposed to 10 μM 4HPR for several days, the cleavage of the hydroxphenyl moiety from the alkyl chain, even if it is inefficient, could lead to generation of sufficient quantities of ATRA or some other retinoid metabolite to induce PrE differentiation. Although there is no direct evidence for such a conversion in F9 cells, our findings are consistent with this interpretation. Further studies are required to determine which of the three explanations for the differentiating activity of 4HPR are true in F9 cells.

As for the mechanism of 4HPR cytotoxicity, much less is known. One promising area of study links this effect to the induction of ROS. It was shown that induction of apoptosis by 4HPR coincided with ROS production in C33A cervical carcinoma cells and that both ROS and apoptosis production could be inhibited by pyrrolidine dithiocarbamate, an oxygen radical scavenger (28). However, appreciable ROS production was not observed in this study in 4HPR-treated F9 cells, using the same assay methods as used for the C33A cells (data not shown). It appears likely that a different mechanism is involved in this case. Another potential mechanism of 4HPR cytotoxicity comes from a study using PC3 prostate cancer cells, in which a rapid induction of TGF-β1 by 4HPR coincided with apoptosis induction (29). It is not known whether the induction of TGF-β1 by 4HPR is dependent on the retinoid receptors in that system. Although TGF-β1 is not expressed at high levels in F9 cells, there is a receptor-dependent induction of TGF-β2 by ATRA in these cells (7). Current experiments are aimed at determining whether 4HPR can regulate TGF-β2 expression in the F9 cells and whether this regulation requires the presence of retinoid receptors.

Fig. 3. 4HPR induces receptor-dependent differentiation of F9 cells. A, F9 WT and AγXα−/− cells were allowed to grow in monolayer culture for 24 h and then treated with 1 μM ATRA, 1 μM 4HPR, or vehicle as control for 4 days with a change of medium and new retinoids added at day 2. Cells were photographed on a phase contrast microscope at ×200 magnification. B, F9 WT and AγXα−/− cells were treated with 1 μM ATRA or 1 μM 4HPR for 4 days, and the expressions of laminin B1 (Ln B1) and type IV collagen (Coll IV) mRNA were determined by RT-PCR.

Fig. 4. 4HPR is an inducer of both cell death and differentiation. Receptor-mediated and receptor-independent effects of 4HPR and ATRA on F9 embryonal carcinoma cells are indicated in relation to time. 4HPR at a low dose (1 μM) can act in a manner similar to 1 μM ATRA to induce differentiation through activation of the nuclear retinoid receptors. At a higher dose (10 μM), 4HPR kills cells within 48 h by inducing both apoptosis and necrosis. The latter effect does not require the presence of the nuclear retinoid receptors.

---

5 J. L. Clifford, unpublished observations.
4HPR is less toxic than other retinoids and shows great promise as a cancer-chemopreventive drug (2, 27). It is currently being tested in a number of chemoprevention trials for breast, prostate, cervical, skin, ovary, and lung cancer as well as transitional cell carcinoma of the bladder (2). Although the current study was conducted with an in vitro system, we would propose that the cancer therapeutic and chemopreventive efficacy of 4HPR in cancer patients might be due to its dual role as both a chemotoxic agent (receptor-independent) and as a retinoid, differentiating agent (receptor dependent). Tumor cells that can escape cytotoxic effects at initial stages of exposure to high doses of 4HPR would then be susceptible to differentiating effects of lower doses of 4HPR. Such cells would be analogous to the 4HPR-resistant F9 cells that we observed after 3 weeks of treatment with 10 μM 4HPR (data not shown). As noted above, only the WT surviving cells exhibited a differentiated morphology, whereas the receptor-deficient cells grew in a manner similar to undifferentiated F9 cell colonies. Because the WT surviving cells were terminally differentiated, they ceased to proliferate and could not be subcloned for further study. However, the 4HPR-resistant, receptor-deficient cells, because they did not differentiate in the presence of 4HPR, continued to grow in colonies and could be propagated. These cells, along with their 4HPR-sensitive parental cell lines, will provide a unique model for the study of the specifically receptor-dependent and -independent effects of 4HPR.

Acknowledgments

We thank Hideki Chiba, Daniel Metzger, and Pierre Chambron for the receptor-null cell lines. We also thank Bristol-Meyers Squibb for permission to use the cell lines.

References

Retinoid Receptor-dependent and -independent Effects of N-(4-Hydroxyphenyl)retinamide in F9 Embryonal Carcinoma Cells


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/1/14

Cited articles
This article cites 25 articles, 15 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/1/14.full#ref-list-1

Citing articles
This article has been cited by 32 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/59/1/14.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.