Mediation of N-(4-Hydroxyphenyl)retinamide-induced Apoptosis in Human Cancer Cells by Different Mechanisms¹

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ABSTRACT

The induction of apoptosis by the synthetic retinoid N-(4-hydroxyphenyl)retinamide (4HPR) has been documented in vitro in various cancer types. A role for reactive oxygen species (ROS) in apoptosis induced by 4HPR in some cancer cells has been demonstrated recently. We studied five different human head and neck and five lung cancer cell lines to determine whether the ROS play a general role in 4HPR-induced apoptosis. We found that 4HPR induced apoptosis in all of the cell lines; however, this effect was blocked by antioxidants in only 2 of the 10 cell lines. 4HPR induced a greater than 4-fold increase in the generation of intracellular ROS in these two cell lines compared with a much lower effect in other cell lines. Furthermore, these two cell lines were most sensitive to the induction of apoptosis by 4HPR. The level of the cellular antioxidant thiol and superoxide dismutase activity were relatively lower in cells, which responded to 4HPR with a high level of ROS generation. These results indicate that although ROS can mediate 4HPR-induced apoptosis in some cells, which may have a low endogenous cellular antioxidant level, other mechanisms exist for 4HPR-induced apoptosis. One such mechanism may involve retinoic acid receptors (RARs) because an RAR antagonist was able to block partially 4HPR-induced apoptosis. In conclusion, 4HPR-induced apoptosis involves at least three different mechanisms, which are complex and can overlap in the same cell line: (a) one mechanism involving 4HPR-induced ROS; (b) one involving RARs; and (c) at least one that does not involve ROS or RARs and remains unclear.

INTRODUCTION

4HPR ³ is effective against breast, prostate, and ovarian cancers in animal models (1–4). This retinoid is being evaluated clinically in the prevention of breast, lung, and head and neck cancer and other malignancies because it has much lower side effects than natural retinoic acids (5–7) and has already shown a potential for preventing ovarian cancer in humans (8). In recent in vitro studies, 4HPR was observed to induce apoptosis in malignant hematopoietic (9), neuroblastoma (10), cervical (11), breast (12), ovarian (13), head and neck (14), and lung (15) cancer cell lines, including those exhibiting resistance to other retinoids.

Presently, the mechanism by which 4HPR induces apoptosis in malignant cells is not well understood. It is also unclear whether the biological effects of 4HPR arise from interaction with nuclear retinoid receptors, which are thought to be the classical pathway of mediating the major effects of retinoids (16). Current experimental data suggest that 4HPR may function by receptor-independent (9, 12, 17) or receptor-dependent mechanisms (18, 19). ROS has recently been found to be involved in the mediation of apoptosis induced by 4HPR in human cervical cancer (20) and leukemia cells (21, 22).

The role of the ROS in mediating apoptosis induced by a variety of agents, including tumor necrosis factor-α, anti-Fas antibodies, some chemotherapeutic agents, and radiation has been well established (23–29). The demonstration of the involvement of ROS in 4HPR-induced apoptosis in cervical carcinoma and leukemia provided a novel mechanism signaling apoptosis in human cancer cells. However, it was not clear whether ROS are also involved in 4HPR-induced apoptosis in other cancer types. Therefore, the present study was performed to determine whether ROS mediate apoptosis induced by 4HPR in human HNSCC and NSCLC cells. We found that ROS play an important role only in specific cell lines that produce high levels of the ROS after exposure to 4HPR, but that the ROS are unlikely to mediate apoptosis induced by 4HPR in most human cancer cells. In addition, we found evidence for the involvement of the RAR-mediated pathway in apoptosis induced by 4HPR in one of these cancer cells.

MATERIALS AND METHODS

Cell Lines. The human NSCLC cell lines SK-MES-1, H226, H460, H1792, and H522 were obtained from the American Type Culture Collection (Rockville, MD) or from Dr. Adi Gazdar (University of Texas Southwestern Medical Center, Dallas, TX), and HNSCC cell lines 17B, 22B, 886, 1483, and SqCCY1 were obtained from Dr. Thomas Carey (University of Michigan, Ann Arbor, MI) and Dr. Peter Sacks (Memorial Sloan Kettering Cancer Center, New York, NY) and Dr. Michael Reiss (Yale University, New Haven, CT). The cells were grown in monolayer culture in a 1:1 (v/v) mixture of DMEM and Ham’s F12 medium containing 5% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Reagents. 4HPR was obtained from Dr. Ronald Lubet (National Cancer Institute, Bethesda, MD). The RARα/β/γ antagonist CD2366 and RARα/β/γ antagonist CD2665 (30) were provided by Dr. Braham Shroot (CIRG/Dalderma, Sophia Antipolis, France). RARα/β/γ antagonist AGN193109 (30) was obtained from Dr. Roshantha A. S. Chandraratna (Allergan Pharmaceuticals, Irvine, CA). The antioxidants BHA and Vit C purchased from Sigma (St. Louis, MO) were used at maximal concentrations that most cell lines can tolerate without cytotoxicity. DCF-DA was purchased from Molecular Probes (Eugene, OR). All other chemicals used in this study were purchased from Sigma.

Cell Survival Assay. The cells were seeded at proper densities that allowed untreated cells to reach a nearly confluent state after a 4-day incubation in 96-well tissue culture plates. After 24 h, the cells were treated with different concentrations of 4HPR alone or in combination with antioxidants or receptor antagonists. Control cultures received the same amount of DMSO or ethanol (0.1%) as the treated cultures did. After 3 days of treatment, cell number was estimated by the SRB assay as described previously (30).

Detection of Apoptosis. After exposure to 4HPR for 24 or 48 h, cells were harvested by trypsinization and counted. One hundred thousand cells and 1 × 10⁶ cells were used for evaluating apoptosis using an ELISA Cell Death Detection kit (Boehringer-Mannheim, Indianapolis, IN) that quantitates the amount of cytoplasmic histone-associated DNA fragments that increases dur-

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¹ The abbreviations used are: 4HPR, N-(4-hydroxyphenyl)retinamide; ROS, reactive oxygen species; RAR, retinoic acid receptor; RXR, retinoid X receptor; SRB, sulfonhydrasine B; DCF-DA, 2',7'-dichlorofluorescein diacetate; SOD, superoxide dismutase; BHA, butylated hydroxyanisole; Vit C, vitamin C; HNSCC, head and neck squamous cell carcinoma; NSCLC, non-small cell lung carcinoma; NBT, nitroblue tetrazolium.

2493
ing apoptosis and using an Annexin V-FITC Apoptosis Detection kit (Oncogene, Cambridge, MA) that detects externalized phosphatidyl serine from the cytoplasmic surface of the cell membrane during apoptosis, respectively, following the manufacturer’s protocols.

Measurement of Intracellular ROS. An equal number of cells (1 × 10^5/well) for each of the cell lines were seeded in 48-well cell culture plates. The intracellular generation of ROS was measured using the oxidation-sensitive fluorescent dye DCF-DA as described previously (22, 31). Briefly, cells were washed twice on the second day after seeding with HBSS (Life Technologies, Inc., Gaithersburg, MD) and incubated with 500 µl of HBSS containing 10 µg/ml of DCF-DA and different concentrations of 4HPR. The cells were then incubated at 37°C, and the fluorescence intensity of dichlorofluorescein was measured at 530 nm emission wavelength after excitation at 480 nm at 30-min intervals for up to 4 h using a CytoFluor 2350 Fluorescence Measurement System (Millipore, Bedford, MA). An increase in fluorescence intensity was used to represent the generation of net intracellular ROS. Four wells were used for each treatment, and the mean ± SD was determined.

Cellular Thiol Content Assay. Cells were seeded in 12-well plates (1 × 10^5 cells/well), and 1 or 5 µM 4HPR was added after 24 h. The total cellular thiol content was determined as described by Kurit and Namiki (32). Briefly, cells were washed twice with PBS 24 h after treatment and lysed with 200 µl/well of a solution containing 1% SDS, 1 mM EDTA, and 50 mM Tris-HCl (pH 8.0). The lysate was dyed with 10 µl of 20 mM 5,5'-dithio-bis(2-nitrobenzoic acid) dissolved in ethanol. Prechilled ethanol (600 µl) was added to the lysate to precipitate the macromolecules, and precipitates were collected by centrifugation at 13,000 × g for 20 min. Absorbance at 410 nm of the supernatant, which was measured with a microtiter reader (Dynetech MR5000, Chantilly, VA), was used to represent the relative cellular thiol content. The data were normalized to cell numbers determined by the SRB assay (30) using a parallel 12-well plate.

Measurement of SOD Activity. After 24 h of 4HPR treatment, cells were washed and then lysed for 15 min on ice with 0.5% NP40 in 50 mM Tris-HCl (pH 7.0). The lysate was centrifuged at 14,000 rpm at 4°C for 15 min, and the supernatant (100 µg) was subjected to electrophoresis on the 7.5% polyacrylamide slab gel under nondenaturing conditions. The SOD activity was determined by the reduction of NBT as described by Peled-Kamer et al. (33). Briefly, the gel was soaked for 1 h in a solution containing 2.45 mM NBT, 28 mM tetramethylenediamine, 0.28 mM riboflavin, and 36 mM potassium phosphate (pH 7.8). SOD activity was observed as a transparent band on the blue background of formazan-stained gel after exposure to light.

RESULTS

Induction of Apoptosis by 4HPR in Human HNSCC and NSCLC Cells. The effects of 4HPR on the survival of human HNSCC and NSCLC cells are presented in Fig. 1. 4HPR exhibited dose-dependent effects on cell survival in all of the cell lines at a concentration range of 1 to 10 µM. Only the NSCLC H522 cells were sensitive to 4HPR at a concentration <1 µM. The 22B cells were the most 4HPR sensitive among the human HNSCC cell lines. The concentration causing cell number decrease by 50% (IC50) after 4HPR treatment was ~0.5 µM in H522 cells but 2–6 µM in other cell lines.

To determine whether 4HPR decreases cell survival through the induction of apoptosis in these cells, we performed an ELISA that quantitates the amount of cytoplasmic histone-associated DNA fragments, which increases during apoptosis. As shown in Fig. 2, 4HPR increased the amount of DNA fragments, i.e., induced apoptosis, in all HNSCC and NSCLC cells. As we found in the cell survival assay, 22B and H522 cells showed relatively higher sensitivity to the induction of apoptosis by 4HPR compared with the other cell lines, especially at the 24-h point. In addition, we confirmed that 4HPR induced apoptosis in some HNSCC and NSCLC cells by Annexin V assay that detects externalized phosphatidyl serine normally localized on the cytoplasmic surface of the cell membrane during apoptosis. Consistent with the results by ELISA, 4HPR induced apoptosis in two of the
NSCLC cell lines (H522 and H460) and two of the HNSCC cell lines (22B and 886; Table 1).

Antioxidant Suppression of 4HPR-induced Apoptosis. 4HPR-induced apoptosis in cervical cancer (20) and leukemia (21, 22) cells could be blocked by antioxidants. If ROS play a critical role in the induction of apoptosis by 4HPR in HNSCC and NSCLC cells, antioxidants would be expected to block 4HPR-induced apoptosis in these cancer cells. Therefore, we analyzed the effects of 4HPR on the survival of 10 human HNSCC and NSCLC cells in the presence of antioxidants. As shown in Fig. 3, the antioxidants BHA and Vit C alone at the indicated concentrations did not affect cell survival, but they reversed the effects of 4HPR on cell survival in 22B and H522 cells. However, in the HNSCC 886 and NSCLC H460 cells (Fig. 3) and in the other HNSCC and NSCLC cells (data not shown), BHA and Vit C failed to block 4HPR-induced apoptosis.

Generation of Intracellular ROS by 4HPR. The finding that the induction of apoptosis by 4HPR in 22B and H522 cells could be suppressed by some antioxidants suggested the involvement of ROS in this apoptosis. Therefore, we measured intracellular ROS generated by exposure of five HNSCC and five NSCLC cell lines to 4HPR. As shown in Fig. 4, 4HPR caused dose-dependent generation of ROS in all of the cell lines. However, a greater than 4-fold increase in the generation of the ROS after exposure to 5 μM 4HPR was observed in 22B and H522 cells compared with only 1-fold or less increase in the other eight cell lines. In H522 cells, 4HPR, even at 1 μM, still stimulated a 4–5-fold increase in ROS generation (Fig. 4).

Antioxidant Suppression of 4HPR-generated ROS. The increase in ROS production induced by 4HPR was suppressed by BHA and Vit C in the two cell lines (22B and H522) with high level of ROS induction and two cell lines (886 and H460) with low level of ROS induction (Fig. 5).

Influence of Endogenous Intracellular Antioxidation Status on Cell Responsiveness to 4HPR. To determine whether differences in the ability of 4HPR to increase ROS in the different cell lines was related to their antioxidation status, we analyzed the cellular thiol content and SOD activity in several NSCLC cell lines. As shown in Fig. 6, H522, the cell line most sensitive to 4HPR, exhibited the lowest total thiol content and SOD activity. The highest level of manganese-SOD and copper zinc-SOD activity was observed in H226 and SK-MES-1 cells, respectively. 4HPR treatment did not change the thiol level and SOD activity, except for an increase in copper zinc-SOD in the H460 cells (Fig. 6B). These data suggested that the high sensitivity of H522 cells to 4HPR may be due to their low antioxidation status.

Table 1 Evaluation of 4HPR-induced apoptosis by Annexin V assay in human NSCLC and HNSCC cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Necrotic cells (%)</th>
<th>Apoptotic cells (early + late) (%)</th>
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<tbody>
<tr>
<td>H522</td>
<td>3.5</td>
<td>20.7 (5.2 + 15.5)</td>
</tr>
<tr>
<td>H460</td>
<td>2.1</td>
<td>11.6 (6.3 + 5.3)</td>
</tr>
<tr>
<td>22B</td>
<td>1.8</td>
<td>11.7 (5.8 + 5.9)</td>
</tr>
<tr>
<td>886</td>
<td>1.0</td>
<td>12.3 (6.3 + 6.0)</td>
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Fig. 3. Antioxidant suppression of the effects of 4HPR on the survival of HNSCC (22B and 886) and NSCLC (H522 and H460) cells. The effects of 4HPR on cell survival were determined by the SRB assay. Columns, mean of four replicate determinations; bars, SD. The concentrations of BHA and Vit C were 50 and 500 μM, respectively. 4HPR was used at a concentration of 0.5 μM (H522), 2.5 μM (22B and H460), and 5 μM (886).

Fig. 4. Generation of intracellular ROS induced by 4HPR in HNSCC (A) and NSCLC (B) cells. Equal numbers of cells from each cell line were seeded in 48-well plates, and intracellular ROS was measured on the second day by the DCF-DA assay. Data points, mean of four replicate determinations; bars, SD. M, DMSO; F, 1.0 μM 4HPR; E, 2.5 μM 4HPR; △, 5.0 μM 4HPR.
Effects of RAR-specific Antagonists on 4HPR-induced Apoptosis. To determine whether the apoptosis induced by 4HPR in human HNSCC and NSCLC cells is mediated by nuclear retinoid receptors, we examined the effects of RAR-specific antagonists on 4HPR-induced apoptosis in some of the HNSCC and NSCLC cells. AGN193109 at a ratio of either 2:1 or 4:1 did not reverse the effects of 4HPR on the survival of H460, H1792, and 1483 cells (data not shown). However, AGN193109 significantly suppressed the effect of 4HPR on the survival of 22B cells to a similar degree as the antioxidants BHA and Vit C (Fig. 7A). Because apoptosis induced by 4HPR
in 22B cells can be suppressed by antioxidants, we determined whether AGN193109 can function as an antioxidant. Using the DCF-DA assay, we established that AGN193109 had no inhibitory effects on the production of ROS by 4HPR in 22B cells (Fig. 8), indicating that AGN193109 does not have antioxidative activity. Because H522 cells are very sensitive to 4HPR, we were able to increase the ratio of antagonists to 4HPR in these cells. As shown in Fig. 7B, a ratio of the RAR pan antagonists CD2366 and AGN193109 and the RAR/¥ antagonist CD2665 to 4HPR of 40:1 still failed to reverse the effect of 4HPR on the survival of H522 cells. Thus, it appears that RAR may play a role in mediating 4HPR-induced apoptosis in 22B but not in H522 cells.

ROS- and Receptor-mediated Pathways May Coexist to Mediate 4HPR-induced Apoptosis in Some Cancer Cells. Because both antioxidants and antagonists each alone only partially blocked 4HPR-induced apoptosis in 22B cells (Figs. 3 and 7A), we concluded that both ROS-mediated and receptor-mediated pathways play a role in 4HPR-induced apoptosis in these cells. To test this hypothesis, we examined the effect of an antioxidant combined with an antagonist on 4HPR-induced apoptosis in 22B cells. The combination of Vit C and AGN193109 significantly blocked 4HPR-induced apoptosis in 22B cells in an additive manner (Fig. 9), indicating that both ROS- and receptor pathways can coexist to mediate 4HPR-induced apoptosis in some cancer cells. However, the fact that even the combination could not completely protect the 22B cells from 4HPR and the inability of antioxidants and antagonists to protect other cells from 4HPR indicate the existence of at least one more mechanism (Fig. 10).

DISCUSSION

It is known that 4HPR can induce apoptosis in a wide variety of cancer cell lines; however, little is known about the mechanism(s) by which this is done. The ability of 4HPR to induce apoptosis in retinoic acid-resistant cells suggests that the mechanism is largely receptor independent. Some studies, however, have shown that RARs appear to be involved in 4HPR-induced apoptosis (18, 19). The present study clearly shows that 4HPR apoptotic effect involves both receptor-dependent (indicated by receptor-antagonist studies) and -independent pathways. The latter pathway includes the recently described ROS as well as an unrelated, unknown RAR-independent mechanism. Therefore, as discussed below, this study shows that 4HPR-induced apoptosis involves at least three different mechanisms, which are complex and can overlap in the same cell line: (a) one mechanism involving 4HPR-induced ROS; (b) one involving RARs; and (c) at least one that does not involve ROS or RARs and remains unclear.

Recently, we (20) and others (22) have demonstrated that ROS are involved in the induction of apoptosis by 4HPR in human cervical cancer and leukemia cells. This finding was important for the understanding of 4HPR apoptotic signaling. However, we did not know whether ROS are the general mediator of apoptosis induced by 4HPR in other human cancer cells (34). This study was designed to address the question of whether ROS generation is the only mechanism by which 4HPR can induce apoptosis. We found that at concentration range of 1–10 m, 4HPR decreased cell survival via induction of apoptosis in all five human HNSCC and five NSCLC cell lines. However, cell death induced by 4HPR could be reversed partially by antioxidants in only two of these cancer cell lines 22B and H522, which were also the cells in which 4HPR increased ROS generation to the highest levels, suggesting that the ROS may play an important role in 4HPR-induced apoptosis only in a few human cancer cells. These cells may be those in which a certain threshold ROS level is reached that results in induction of apoptosis by 4HPR as suggested by our previous work (20).

Under normal circumstances, cells are able to balance the production of oxidants (e.g., ROS) and antioxidants such as glutathione, SOD, catalase, and glutathione peroxidase, resulting in a redox equilibrium (35). If the antioxidant defense becomes overwhelmed by uncontrolled levels of ROS, extensive oxidation of cellular components, protein degradation, disruption of intracellular Ca2+ homeostasis, and DNA damage may lead to cell death (36). In our study, we found that 4HPR induces a high level of ROS production in some cells

Fig. 10. Schema of the mechanisms by which 4HPR can induce apoptosis. Apoptosis induced by 4HPR can be mediated by ROS production (A), the RAR/RXR pathway (B), and unknown mechanisms (C).
MECHANISMS OF 4HPR IN INDUCTION OF APOPTOSIS

but not in others. One possible reason for this is that there may be an abnormal antioxidant defense system in cells that produce a high level of the ROS when exposed to 4HPR. Our results show that indeed H522 cells have the lowest thiol level and SOD activity, indicating that the endogenous antioxidant defense in this cell line is lower than in the other cells. We propose that the high level of ROS generation by 4HPR and supersensitivity to 4HPR-induced apoptosis in H522 cells may be associated with their low antioxidant status.

Many retinoids act by activating gene transcription following their binding to nuclear receptors, which include RARα, RARβ, RARγ, and RXRα, RXRβ, and RXRγ (16). Recently, 4HPR has been reported to activate transcription of retinoic acid response elements, most probably through RAR/RXR heterodimers (18, 19). In the present study, several RAR-specific antagonists such as AGN193109, CD2366, and CD2665 did not suppress the effect of 4HPR on the survival of several cell lines, but they did partially reverse the effect of 4HPR on the survival of one cell line (22B). These results suggest that both RAR-independent and RAR-dependent pathways are involved in mediating apoptosis induced by 4HPR, depending on the cell lines. The fact that 4HPR-induced apoptosis in 22B cells can be inhibited by the combination of an antioxidant and an antagonist in an additive manner indicates that both pathways may mediate apoptosis induced by 4HPR in the same cells.

In addition to the participation of the ROS and RARs in mediating apoptosis induced by 4HPR, other pathways may also be involved, because apoptosis induced by 4HPR in some cell lines, such as 1483, H460, and H1792, cannot be blocked by either antioxidants or RAR antagonists. Therefore, we propose that different mechanisms are involved in the mediation of 4HPR-induced apoptosis, depending on the cell lines as summarized in Fig. 10. The elucidation of these mechanisms by which 4HPR induces apoptosis in different cancer cells will be helpful for better understanding new apoptotic signaling pathways of retinoids and will benefit the clinical application of 4HPR in the prevention and treatment of cancer.

REFERENCES

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