Differential Induction of Apoptosis by all-trans-Retinoic Acid and N-(4-Hydroxyphenyl)retinamide in Human Head and Neck Squamous Cell Carcinoma Cell Lines

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ABSTRACT

Retinoids have been shown to act as cytotatic agents against a variety of tumor cell types, including squamous carcinoma cells. Recently it was reported that certain retinoids can induce apoptosis as well. Because we are investigating the potential of retinoids in chemoprevention and therapy for head and neck premalignant and malignant lesions, we compared the effects of all-trans-retinoic acid (ATRA) and N-(4-hydroxyphenyl)retinamide (4HPR) on seven human head and neck squamous cell carcinoma cell lines (17A, 17B, 22A, 22B, 38, SqCC/Y1, and 1483). Six of the seven cell lines showed dramatic morphological changes after treatment with 10 μM 4HPR, whereas no such changes were induced by 10 μM ATRA. To determine whether these retinoids can induce apoptosis, we analyzed both detached and attached cells after 2, 5, and 7 days of treatment for evidence of DNA fragmentation by DNA electrophoresis on agarose gels. In five of the seven cell lines, a DNA ladder was observed after treatment with 10 μM 4HPR for 5 or 7 days, whereas treatment with ATRA resulted in a less pronounced effect. In 17B cells, a clear DNA ladder was observed as early as 2 days after treatment with 4HPR; however, neither ATRA nor 9-cis-retinoic acid was as effective. In addition, morphological changes associated with apoptotic cell death, such as chromatin condensation and nuclear segmentation, were observed by propidium iodide staining and by electron microscopic analysis after 4HPR treatment. These results demonstrate that 4HPR causes apoptosis in several head and neck squamous cell carcinoma cell lines and that it is more potent in this effect than ATRA.

INTRODUCTION

In 1995, cancers of the head and neck were estimated to account for about 3.2% (n = 39,750) of new cancer cases and 2.25% (n = 12,460) of cancer deaths (1). SCC is the major cancer in the head and neck region. Human HNSCCs are generally treated by surgery and/or radiation therapy. However, the overall survival rate (approximately 45%) among HNSCC patients has remained unchanged for the last three decades, despite adjuvant chemotherapy. The most frequent treatment failures in patients who have been treated curatively for HNSCC occur locally, including regional recurrence and second primary tumors, which are considered to be the major cause of death in these patients (2). For these reasons, new approaches to the prevention of head and neck cancer primary and second primary tumors are being explored (2, 3). Chemoprevention with retinoids (vitamin A analogues) is now highlighted as a method that has the potential to reduce the incidence of and mortality from head and neck cancers (3). Retinoids suppress the proliferation of HNSCC cells in monolayer cultures, inhibit the formation of SCC colonies in semisolid agarose, and decrease the growth of HNSCC multicellular spheroids. In addition, retinoids suppress the expression of several squamous differentiation markers and inhibit cornified envelope formation (4). 13-cis-Retinoic acid and 4HPR have been reported to suppress oral premalignant lesions (e.g., leukoplakia) that may progress to SCC (5, 6). The mechanism(s) of these clinically significant activities of retinoids are still unknown.

4HPR was found to be effective in preventing the development of a variety of cancers in experimental animals, including breast, bladder, lung, and prostate cancer (7–12). Preventative as well as therapeutic effects of 4HPR have been reported against breast, prostate, and ovarian cancer in rodents (13–15). Some activity of 4HPR in humans has already been reported in preneoplastic oral leukoplakia by systemic and topical treatment (16–18) and in decreasing the incidence of ovarian cancer among breast cancer patients (19). Various clinical chemoprevention trials targeting breast, prostate, cervix, skin, and lung are in progress (reviewed in Refs. 6, 20, 21).

Little is known about the mechanism of 4HPR action at the cellular or molecular level. 4HPR was found to suppress colony formation in semisolid agar in vitro by cells dissociated from fresh human tumors, suggesting a direct effect on the tumor cells (22). Induction of apoptosis by 4HPR has been reported in malignant hematopoietic cell lines (23) and in neuroblastoma cell lines (24, 25). ATRA also induced apoptosis in leukemia and neuroblastoma cells (26–28), as well as in the normal mouse limb bud (29). In these cases, tissue transglutaminase seems to play an important role (28–32). Suppression of bcl2 by

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ATRA in leukemia cells has also been suggested to mediate apoptosis (33). There have been no reports that 4HPR binds to any of the known retinoid receptors. Furthermore, 4HPR induced apoptosis in ATRA-resistant mutant HL-60 leukemia cells (23), suggesting that it is likely working through a pathway distinct from that of ATRA.

First described in 1972 by Kerr et al. (34), apoptosis is a physiological form of cell death by the active regulatory process of eliminating cells for homeostatic regulation of tissue mass as a counterbalance to cell division during development and cellular immune responses. Dysregulation of apoptosis may lead to altered cell number within the tissue and, finally, to malignant transformation.
transformation. This process, which is characterized by cell shrinkage, chromatin condensation, nuclear segmentation, and internucleosomal degradation of DNA (34), is initiated in response to a variety of external and intracellular signals (35).

Our interest in exploring the potential use of retinoids for the prevention and treatment of head and neck cancer has led us to try to determine whether ATRA and 4HPR can induce apoptosis in HNSCC cell lines. This study has shown that 4HPR is a potent inducer of apoptosis in such cells and suggests that the potential of this compound in the prevention and treatment of head and neck cancer should be investigated further.

MATERIALS AND METHODS
Cell Culture and Treatment with Retinoids. The HNSCC cell lines UMSCC-17A, UMSCC-17B (36), UMSCC-22A, UMSCC-22B (37), and UMSCC-38 (38) were obtained from Dr. T. Carey (University of Michigan, Ann Arbor, MI). 1483 cells (39) and SqCC/Y1 cells (40) were obtained from Dr. P. G. Sacks (Memorial Sloan-Kettering Cancer Center, New York, NY) and Dr. Michael Reiss (Yale University, New Haven, CT), respectively. The cells were maintained in a 1:1 mixture of DMEM:Ham’s F12 medium supplemented with 10% fetal bovine serum. 4HPR was obtained from Dr. Ronald Lubet (Division of Cancer Prevention and Control, National Cancer Institute, Bethesda, MD) via Ogden Bioservices (Rockville, MD). ATRA and 9CRA were obtained from Dr. Werner Bollag (F. Hoffmann-La Roche, Basel, Switzerland). The retinoids were dissolved in DMSO at a concentration of 10 μM and diluted in growth medium immediately before addition to cell cultures. Control cultures received the same amount of DMSO as the treated cultures.

DNA Extraction and Gel Electrophoretic Analysis of DNA Fragmentation. Soluble DNA was extracted as described previously (41). Briefly, after 2, 5, and 7 days of incubation with DMSO only, 10 μM ATRA, or 5 or 10 μM 4HPR, the cells floating in medium were collected by centrifugation, and the cells that remained attached to the dish on day 7 were detached by trypsinization. The cells were centrifuged into a pellet and resuspended in Tris-EDTA buffer (pH 8.0). The plasma membrane of the cells was lysed in 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% Triton X-100 on ice for 15 min. The lysate was centrifuged at 12,000 × g for 15 min to separate...
soluble (fragmented) DNA from pellet (intact genomic) DNA. Soluble DNA was treated with RNase A (50 µg/ml) at 37°C for 1 h, followed by treatment with proteinase K (100 µg/ml) in 0.5% SDS, at 50°C for 2 h. The residual material was extracted with phenol/chloroform, precipitated in ethanol, electrophoresed on a 2% agarose gel, and stained with ethidium bromide. The loading amount on the gel was adjusted to the cell number before the treatments. The gels were then photographed in the dark under UV illumination.

Detection and Quantitation of Apoptosis by Propidium Iodide Staining of Adherent Cells. After 2 or 5 days of culture, the cells were fixed with 4% paraformaldehyde in 0.1% phosphate buffer (pH 7.4) for 10 min at room temperature, followed by 70% ethanol containing 1% HCl for 10 min at −20°C, and then stained with 1 µg/ml propidium iodide containing 100 µg/ml DNase-free RNase A for 30 min at 37°C to visualize the nuclei (42). The cells were examined with a fluorescence microscope and photographed with Kodak TMax 400 film. More than 1000 cells in several microscopic fields were counted under the fluorescence microscope to determine the percentage of apoptotic cells.

Electron Microscopic Analysis of Apoptotic Cells. After 4 days of culture, the cells were harvested and fixed with 2% glutaraldehyde in PBS for 30 min at 4°C, washed in PBS, fixed again with 1% OsO4 in PBS, washed again, and dehydrated by placing in increasing concentrations of ethanol (50% to 100%). The cells were then embedded in araldite resin, following the standard procedure (43), and after the polymerization of the blocks, ultrathin sections were prepared with a Reich-Jung ultramicrotome. Sections were stained for 10 min with 2% uranyl acetate followed by staining with lead citrate. Sections were viewed and micrographs were taken with a Philips electron microscope.

RESULTS

Differential Effects of ATRA and 4HPR on the Morphology of Human HNSCC Cell Lines. We used seven HNSCC cell lines established from tumors in distinct regions of the head and neck (Table 1) to investigate the effects of ATRA and 4HPR on cell growth. Treatment of 80% confluent cultures of the HNSCC cell lines for 7 days with 10 µM ATRA caused only minor changes in cell morphology and density relative to the control (Fig. 1). In contrast, a similar treatment with 4HPR resulted in drastic morphological changes, decreased cell density, and, in some cells (22B, 38, and 1483), cell rounding and detachment (Fig. 1). Cells from cell lines 22A, 22B, and 38 exhibited focal stratification, which was inhibited by the 7-day ATRA treatment (Fig. 1). Treatment with 10 µM 9CRA had the same effect as 10 µM ATRA on cell growth and morphology (data not shown). Three of the seven cell lines (22B, 38, and 1483) were very sensitive to 4HPR and exhibited reduced growth and changes in morphology already after a 3-day treatment, whereas the same concentration of ATRA had little effect on the growth and morphology of these cells at that time (data not shown). In these three cell lines, the effect of 5 µM 4HPR was also considerable, albeit less than that of the 10 µM concentration (data not shown).

Table 1
HNSCC cell lines used in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Sex/age (years) of patient</th>
<th>Location of tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>17A*</td>
<td>F/48</td>
<td>Larynx</td>
</tr>
<tr>
<td>17B*</td>
<td>F/48</td>
<td>Lymph node metastasis</td>
</tr>
<tr>
<td>22A*</td>
<td>M/53</td>
<td>Hypopharynx</td>
</tr>
<tr>
<td>22B*</td>
<td>M/53</td>
<td>Lymph node metastasis</td>
</tr>
<tr>
<td>38</td>
<td>M/64</td>
<td>Tonsillar fossa</td>
</tr>
<tr>
<td>1483</td>
<td>M/66</td>
<td>Retromolar trigone</td>
</tr>
<tr>
<td>SqCC/Y1</td>
<td>Unknown</td>
<td>Buccal mucosa</td>
</tr>
</tbody>
</table>

* 17 and 17B cell lines were derived from the same patient.
+ 22A and 22B cell lines were derived from the same patient.

Induction of Apoptosis by 4HPR in HNSCC Cell Lines: DNA Ladder Formation. The rounding and detachment of cells in some of the cell lines after treatment with 4HPR raised the possibility that the cells were undergoing apoptosis. Because apoptosis is associated with the activation of an endonuclease that degrades DNA into large fragments of random size and smaller fragments that are multiples of 180 base pairs formed by cuts between nucleosomes, we analyzed the soluble DNA fraction from the detached (floating) cells after 2, 5, and 7 days and the cells that remained attached after 7 days by gel electrophoresis. We found evidence for apoptosis in the form of the characteristic regularly spaced ladder of DNA fragments (44) in most floating cells in cultures treated with either ATRA or 4HPR (Fig. 2). The intensity of the ladder increased from 2 to 7 days and from 5 to 10 µM 4HPR. ATRA was ineffective at 5 µM and 4HPR was ineffective at 1 µM (data not shown). With the exception of the SqCC/Y1 and the 1483 cells, 4HPR was more potent than ATRA in inducing DNA ladder formation, as indicated by the more intensely stained DNA ladders. The differential effect of these two retinoids was most prominent in the 17A and 22B cells, in which the DNA ladder was observed in the floating cells from cultures treated with 10 µM 4HPR, whereas ATRA had no or minimal effect. In the 17B cells, which were derived from a lymph node metastasis of the primary laryngeal carcinoma 17A, the ladder observed in floating cells from cultures treated with 10 µM 4HPR was more intense than in cultures treated with 10 µM ATRA, whereas the effect of 5 µM 4HPR was similar to that of 10 µM ATRA. In the 22A cells, DNA ladder formation was observed only after a 7-day treatment with ATRA or 4HPR, and the two retinoids exhibited a similar effect. In contrast, in the 22B cells, the ladder was observed primarily in cultures treated with 4HPR. The SqCC/Y1 cells exhibited a certain level of DNA ladder in untreated cultures, which was not increased markedly after treatment with either retinoid. The 1483 cells showed marked sensitivity to both retinoids, although the DNA ladder observed in the 4HPR-treated cultures was more intense. In summary, in five of seven cell lines, DNA ladder formation was more extensive in cultures treated with 10 µM 4HPR than with the same concentration of ATRA. Although in four of seven cell lines ATRA treatment increased the amount of soluble DNA in comparison with the control, in most cases, the DNA ladder was not as well defined as in the 4HPR-treated cells.

Although formation of a DNA ladder is considered to be one of the most characteristic indicators of apoptosis (44), there are limitations in determining the extent of apoptosis with this
that cannot be precipitated with ethanol. This results in a low control cultures (as shown in Fig. I).

During treatment with apoptosis-inducing agents compared to the following reasons:

a) The total number of cells decreases during treatment with apoptosis-inducing agents compared to control cultures (as shown in Fig. 1). Consequently, an agent like 4HPR that induces apoptosis after a short exposure time causes a marked reduction in cell number and appears to be less effective after a longer treatment because fewer cells remain that can yield apoptotic cells. b) Fragmented DNA in apoptotic cells may undergo further degradation into small oligonucleotides that cannot be precipitated with ethanol. This results in a low yield of soluble DNA and consequently a weak ladder on an agarose gel. In our studies, this could have led to an underestimation of the higher potency of apoptosis induction by 4HPR compared to ATRA in nearly all of the HNSCC cell lines, which show fewer adherent cells remaining after 7 days of treatment (Fig. 1).

Quantitation of Apoptosis by Analysis of Propidium Iodide-stained Nuclei. When cells are stained with propidium iodide and observed under a fluorescence microscope, normal nuclei appear as homogeneously fluorescent oval discs; in contrast, the nuclei of apoptotic cells appear as condensed, often fragmented, intensely fluorescent discs (42). Using this staining method, we found that the 17A cells contained fragmented nuclei characteristic of apoptotic cells after 2 days of treatment and more so after 5 days with 4HPR, whereas the nuclei of control cells did not display such features (Fig. 3). The degree of apoptosis induced by 4HPR in the 17A and 17B cells was quantitated by counting the number of apoptotic nuclei among 1000 nuclei (Fig. 4). More apoptotic nuclei were observed in 17A than in 17B cells. The percentages of apoptotic cells in 17A cells after 2-day and 5-day treatment with 4HPR were 10.4 ± 1.4% and 24.3 ± 5.9%, respectively, whereas those in the control 2-day and 5-day cultures were less than 1%. In 17B, 3.7 ± 0.6% and 4.7 ± 1.3% of the attached cells showed apoptotic features after 2 and 5 days of 4HPR treatment, respectively. Like the other methods, this one also has some limitations in that it does not include floating cells. Therefore, these values are an underestimation of the actual degree of apoptosis.

Electron Microscopic Evidence of Induction of Apoptosis in HNSCC Cells by 4HPR. Changes characteristic of apoptotic nuclei such as chromatin condensation and fragmentation can be observed by electron microscopy and are considered to be the "gold standard" of apoptosis. Electron microscopic analysis of the 22A cells revealed gross changes induced by 10 μM 4HPR in the nuclei and cytoplasm of apoptotic cells. Representative photomicrographs showing an untreated cell with a normal nucleus and an apoptotic cell with chromatin condensation, peripheral chromatin "caps," and blebbing into the cytoplasm are shown in Fig. 5.

DISCUSSION

Activation of apoptosis in cells at risk for neoplastic transformation may constitute a physiological antineoplastic mechanism. Apoptosis of DNA-damaged cells could protect the organism from cancer development by eliminating cells that might otherwise replicate the damaged DNA, a process that could lead to mutations and eventually to cancer (52). For example, the maturation of keratinocytes is a paradigm of cells engaged in an asynchronous apoptotic program (53). Dysregulation of this program by aberrant differentiation and loss of ability to undergo a physiological cell death may lead to the development of SCCs. Agents that can induce or restore the ability to undergo apoptosis in premalignant and malignant cells are expected, therefore, to be effective in cancer prevention and treatment (54). In this context, it is of interest that although most of the reports on the effects of retinoids on tumor cells in vitro described cytoplastic effects (e.g., accumulation of cells in the G1 phase of the cell cycle) in monolayer cultures and suppressed colony formation in semisolid medium (55), both 4HPR (23–25) and ATRA (26–33) were also found to induce apoptosis in mesenchymal, neuroectodermal, hematopoietic, and epithelial...
Fig. 3 Detection of apoptotic cells by propidium iodide staining among adherent HNSCC 17A cells treated with 4HPR. After treatment with DMSO (left column, A and C) or 10 μM 4HPR (right column, B and D) for 2 days (top row, A and B) or 5 days (bottom row, C and D), the cells were stained with propidium iodide containing DNase-free RNase A and observed and photographed under a fluorescence microscope. Apoptotic nuclei appear in B and D as condensed, brightly fluorescent, fragmented particles that are easily distinguished from the intact normal nuclei (A and C).

This report is the first to describe the ability of both ATRA and 4HPR to induce apoptosis in HNSCC cell lines; a previous report presented some evidence for the induction of cell death in a multicellular spheroid of a HNSCC cell line by ATRA (56). We have demonstrated that 4HPR induced apoptosis in most of the HNSCC cell lines we tested using both biochemical and morphological methods. In six of seven HNSCC cell lines, changes in morphology induced by 4HPR treatment were more drastic than those induced by ATRA or 9CRA. The degree of 4HPR-induced changes in morphology varied among the HNSCC cell lines. Because some cell lines did not show marked suppression of growth, we suggest that even when used at high dosages, 4HPR does not cause a general cytotoxicity. 17A cells exhibited changes in morphology and DNA ladder formation after several days of exposure to 4HPR, but these cells did not respond to ATRA. In contrast, 17B cells, which were derived from a metastasis of 17A and should have almost the same genetic background, exhibited a distinct pattern of response in that they did not show marked changes in morphology but exhibited marked DNA fragmentation within a 2-day exposure to 4HPR. This rapid apoptosis might be independent of differentiation. Previous studies indicated that the ability of ATRA to induce apoptosis in human cervical carcinoma or neuroblastoma was independent of the neuronal differentiation induced by ATRA in the neuroblastoma cultures (28). In contrast, ATRA enhanced apoptosis in HL-60 myeloid leukemia cells 6–8 days subsequent to inducing cell differentiation into neutrophil-like cells (26). Apoptosis induction by 4HPR in several human cells during normal development and in cultured untransformed and tumor cells.
lymphoma and leukemia cells was independent of differentiation because 4HPR induced the characteristic fragmentation of DNA as soon as 6 h after treatment (23). Furthermore, 4HPR induced apoptosis in ATRA-resistant mutant HL-60 leukemia cells, suggesting a mechanism of action that may be distinct from that of ATRA (23). We found that the HNSCC 22B cell line was not the most responsive to the induction of apoptosis by 4HPR among the HNSCC cell lines we examined, although this cell line was the most sensitive to ATRA in growth inhibition. Furthermore, some cell lines (e.g., 17A and 22B) were resistant to ATRA but were responsive to 4HPR treatment. Thus, our results also demonstrate that 4HPR is much more potent than ATRA in inducing apoptosis in HNSCC cells and further support the hypothesis that the two retinoids act by distinct mechanisms.

The mechanism by which ATRA induces apoptosis is not understood. ATRA-induced apoptosis in mouse limb bud core mesenchymal cells was linked to modulation of gene expression and induction of nuclear retinoic acid receptor β (57). ATRA-induced apoptosis has also been associated with an increase in tissue transglutaminase in various malignant cell types (30–32) and with suppression of the level of p26-Bcl-2 protein in leukemia cells (33). Nuclear retinoic acid receptors, the presumed mediators of most retinoid actions on gene expression and changes in cell phenotype (58–60), are expressed in HNSCC cell lines in vitro (61) and in HNSCC tumors in vivo (62). However, it is not clear whether they also mediate the induction of apoptosis by ATRA. Furthermore, our finding that ATRA and 9CRA, which bind to the nuclear receptors, are not as effective in inducing apoptosis in HNSCC as 4HPR, which cannot bind to the receptors, suggests that the retinoic acid receptor β(retinoid X receptor-mediated pathway may not be involved in 4HPR-induced apoptosis.

4HPR has been found to be an effective chemopreventive agent in experimental animals (7–14) and has exhibited some efficacy in clinical trials of cancer prevention (6, 15–20); however, there is only a limited understanding of the mechanism of action of this retinoid. Likewise, although 4HPR induces apoptosis in various cells (23–25) there are, at the present time, no clues to the mechanism underlying this effect. Eliminating tumor cells by inducing apoptosis is a recognized effect of many chemotherapeutic agents (63). Therefore, our demonstration that 4HPR can induce apoptosis in malignant HNSCC cells indicates that it may have some effectiveness in cancer therapy. If this retinoid can also induce apoptosis in premalignant cells, it may cause the elimination of the preneoplastic lesion. Previous clinical trials have shown that 4HPR is less toxic than most other retinoids (64). Its ability to induce apoptosis more effectively than other retinoids suggests that its potential for the prevention and treatment of head and neck cancer should be investigated further.

REFERENCES

Differential induction of apoptosis by all-trans-retinoic acid and N-(4-hydroxyphenyl)retinamide in human head and neck squamous cell carcinoma cell lines.

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