A Novel Mechanism by Which N-(4-hydroxyphenyl)retinamide Inhibits Breast Cancer Cell Growth: The Production of Nitric Oxide

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Abstract

N-(4-Hydroxyphenyl)retinamide (4-HPR) induces apoptosis in breast cancer cells; however, the molecular basis by which 4-HPR induces apoptosis is not well understood. In breast cancer cells, nitric oxide (NO) is predominantly an apoptotic inducer. Apoptotic agents, such as phorbol ester, tumor necrosis factor-α, and peptide hormones, have been shown to increase NO production in breast cancer cells. Therefore, we hypothesized that the production of NO is vital for 4-HPR to induce apoptosis in breast cancer cells. We found that 4-HPR induced NO production in a dose-dependent manner in all of the breast cancer cell lines tested. The degree of growth inhibition and apoptotic induction by 4-HPR was directly correlated with the amount of NO produced. To prove that NO is essential for 4-HPR to induce apoptosis, breast cancer cells were cocultivated with a competitive NO synthase (NOS) inhibitor, Nω-monomethyl-L-arginine (L-NMMA), and 4-HPR. L-NMMA prevented 4-HPR from inducing inhibitory effects, indicating that NO is crucial for 4-HPR to induce its apoptotic effects in breast cancer cells. IFNs and tamoxifen (TAM) have been shown to potentiate 4-HPR effects in breast cancer cells. Both IFN-γ and TAM enhanced the ability of 4-HPR to induce NO production in breast cancer cells, which was correlated with increased apoptosis. Alone, 4-HPR increased expression of both inducible NOS (NOSII) and endothelial NOS (NOSIII). When combined with 4-HPR, IFN-γ and TAM enhanced NOSII expression. Thus, we have identified a novel mechanism by which 4-HPR induces apoptosis in breast cancer cells, i.e., by increasing NOS expression to induce NO production.

Introduction

4-HPR, a synthetic derivative of all-trans retinoic acid, exhibits apoptotic and anti-invasive effects in breast cancer cells in vitro (1–4). 4-HPR also inhibits carcinogen-induced mammary cancer in animal models (5). Furthermore, 4-HPR accumulates selectively in breast tissue, and this may account for its reduced toxicity in comparison to other retinoids (6–8). Clinically, 4-HPR effectively reduces local recurrent and contralateral breast cancer in premenopausal women (9, 10). However, the use of 4-HPR in clinical trials in women with advanced breast cancer has shown only limited success (11). Other agents have been combined with 4-HPR to improve its clinical use in breast cancer chemoprevention and treatment. IFNs have been shown to potentiate retinoid-induced growth inhibition in breast cancer cells in vitro (12, 13). TAM has been found to increase 4-HPR-induced growth inhibition in vitro and in vivo (14–16). Clinical data collected thus far indicate that combined 4-HPR and TAM may have potential chemopreventive effects in ER+ breast cancer patients (17) and therapeutic effects for patients with ER− metastatic breast cancer but not for those with ER− metastatic disease (18). Yet, the molecular mechanisms by which 4-HPR exerts its apoptotic effects in breast cancer cells as well as the mechanisms by which IFNs and TAM enhance the potency of 4-HPR are not well understood.

Unlike typical retinoids, 4-HPR appears to induce its apoptotic effects via retinoid receptor-independent mechanisms (1, 19–21). 4-HPR has been shown to reduce telomerase activity (22, 23) and insulin growth factor-1 production (24, 25) and to elevate transforming growth factor-β production (3); whether these observations are the mechanisms or the results of 4-HPR-induced apoptosis are not known. Recent reports suggest that 4-HPR-induced apoptosis may be mediated through increases in free radicals (26), reactive oxygen species (27–30), mitochondrial permeability transition (31, 32), ceramide levels (33, 34), and caspase 3 activity (30, 35). Whether 4-HPR uses these or some other mechanisms to induce apoptosis in breast cancer has not been fully investigated.

NO has been shown to inhibit proliferation and induce apoptosis in breast cancer cells (36–40). NO is a free radical synthesized from arginine by three different isoforms of NOS: NOSI, NOSII, and NOSIII. The constitutive isoforms NOSI and NOSIII are calcium and calmodulin dependent, whereas the inducible isoform NOSII is calcium and calmodulin independent. Increased NO production has been reported in...
breast cancer cells treated with various apoptotic agents, such as tumor necrosis factor-α, phorbol ester, and peptide hormones (36, 38, 41). Recently, the antiproliferative effects of all-trans retinoic acid have been correlated with increased NO production in a breast cancer cell line (42).

On the basis of this information, we hypothesized that one mechanism by which 4-HPR exerts its growth-inhibitory effects on breast cancer cells is by inducing NO production. In this study, we determined the effects of 4-HPR alone and in combination with IFN-γ and TAM on the growth and the production of NO in breast cancer cells. Data reported here demonstrate that 4-HPR induces NO production in breast cancer cells and that NO production is essential for 4-HPR-induced inhibition. We further demonstrated that one potential mechanism by which IFN-γ and TAM enhance the potency of 4-HPR in breast cancer cells is by increasing NO production.

Materials and Methods

Reagents. 4-HPR, IFN-γ, TAM citrate, and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). NOSII and NOSIIIB antibodies were purchased from BD Transduction Laboratories (San Diego, CA). Vectastain Elite ABC (avidin-biotin-peroxidase complex) kit, hematoxylin, and 3-amino-9-ethyl-carbazole were purchased from Vector Laboratories (Burlingame, CA). Aqua-Mount was purchased from Lerner Laboratories (Pittsburgh, PA). Stock solutions (10 mM) of 4-HPR and TAM were prepared in PBS and stored at 4°C acetone and stored at 20°C. IFN-γ was prepared in PBS at a concentration of 1 μg/ml and stored at −20°C. L-arginine was purchased from Alexis Biochemicals (San Diego, CA). Stock solutions (10 mM) of L-arginine were prepared in PBS and stored at 4°C. All of the reagents were diluted in culture medium to the indicated final concentration.

Cell Lines and Culture Conditions. ER+ breast cancer cell lines (MCF-7 and T47D) and ER- breast cancer cell lines (MDA-MB-453 and SKBr-3) were obtained from American Type Cell Culture (Manassas, VA). The cells were cultured in DMEM/F12 medium supplemented with 5% heat-inactivated FBS at 37°C under 5% CO2 in a humidified incubator.

Cell Growth and NO Assay. Breast cancer cells were plated at 1 × 10^6 cells/well in 6-well plates in 2 ml of DMEM/F12 medium supplemented with 5% FBS. Twenty-four h later, cells were treated with 4-HPR (1, 2.5 μM), IFN-γ (25, 50, 100 IU/ml), or TAM (0.1, 1 μM). For combination treatments, a 1-μM dose of 4-HPR was used. After 5 days of incubation, cell growth was determined by total live cell counts using trypan blue exclusion. Supernatants were collected from treated cells, and aliquots were stored at −80°C for NO determination. Total NO was determined by quantifying nitrite, the stable end product of NO oxidation. Nitrite accumulation was determined spectrophotometrically using a Colorimetric Non-enzymatic Nitric Oxide Assay kit (Oxford Biomedical Research, Oxford, MI). Briefly, 100-μl samples were incubated with 0.5 g of cadmium beads overnight. Cadmium was used to catalyze the reduction of nitrate to nitrite, thus allowing for the measurement of total NO present in the samples. The samples were reacted with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylene diamine), and the absorbance was measured at 540 nm in a Microplate reader (Molecular Devices, Sunnyvale, CA). Sodium nitrite was used as a standard. Nitrite values were normalized for total cell counts and expressed as μM per 1,000,000 cells. Values are reported as means ± SD for experiments performed in triplicate.

Western Blot. Protein lysates (50 μg) from untreated exponentially growing MCF-7, T47D, SKBr-3, and MDA-MB-453 cells were loaded on a 12% polyacrylamide gel to determine ERα status in these breast cancer cell lines. Proteins were electrophoresed and electrotransferred as described by Tari et al. (43). Membranes were incubated with monoclonal ERα mouse antibody (Novacastra Laboratories Ltd., Burlingame, CA). Protein bands were visualized by enhanced chemiluminescence (Kirkgaard and Perry Laboratories, Gaithersburg, MD). Images were scanned using an Alpha Imager application program (Alpha Innotech, San Leandro, CA).

Apoptosis Analysis. The effect of 4-HPR alone and in combination with IFN-γ and TAM on apoptosis was analyzed by flow cytometry. Approximately 1 × 10^6 T47D cells were trypsinized, collected by centrifugation at 1500 rpm for 5 min, washed in PBS, and resuspended in 1 ml of PBS. The cell suspension was added to 1 ml of cold 70% ethanol and incubated overnight at −20°C. Cells were centrifuged at 1500 rpm for 10 min at 4°C and then washed twice in PBS, and the pellet was left loose. Approximately 0.5–1 ml of PBS containing RNase (20 μg/ml) and propidium iodide (50 μg/ml) was added to each cell pellet, followed by 20 min of incubation at room temperature. Flow cytometric analysis was performed using a Coulter Epics Profile 488 laser. Supernatants from treated cells were collected for subsequent NO determination as described above.

Inhibition of NO Production. To determine the importance of NO production in 4-HPR-induced growth inhibition in breast cancer cells, the competitive NOS inhibitor L-NAME was used to block NO synthesis. T47D and MCF-7 cells were plated (1 × 10^5 cells/well) in 6-well plates in 2 ml of DMEM/F12 medium supplemented with 5% FBS. The next day, cells were treated with 4-HPR (1 μM) in the absence and presence of L-NAME (1, 10, 100 μM) for 5 days. After incubation, cell growth and NO production were determined as described above.

Immunohistochemical Staining. T47D cells (1 × 10^5 cells) were plated in 6-well plates in DMEM/F12 medium supplemented with 5% FBS and treated with 4-HPR alone (1, 2.5 μM) or in combination with IFN-γ (50 IU/ml) or TAM (1 μM). Cells were incubated with 1 μM 4-HPR alone or in combination with TAM for 5 days. Cells were treated with 2.5 μM 4-HPR or a combination of 4-HPR and IFN-γ for 3 days. Cells were harvested and suspended in PBS (1 × 10^6 cells/ml). Cytospins for each treatment were prepared by using 100 μl of the appropriate cell suspension. Slides were quick-fixed in −20°C acetone and stored at −20°C until immunostaining. Slides were prepared according to the protocol of Ekmeckcioglu et al. (44). Briefly, slides were fixed in acetone at −20°C and then incubated in 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. The slides were incubated in PBS containing 0.05% Triton X-100.
to permeabilize the cells. An ABC kit (Vectastain Elite) was then used to detect the primary antibody staining. The immunostaining was developed using 3-amino-9-ethyl-carbazole as a chromagen. Slides were counterstained with hematoxylin and mounted with Aqua-Mount. The slides were analyzed for both percentage and intensity of immunolabeling. The intensity of NOS immunostainings was evaluated by dividing the cytoplasmic staining reaction into four groups: −, none; +, light; ++, moderate; and ++++, intense. Percentages of immunostainings were evaluated as follows: −, <5% positive cells; +, 5–25%; ++, 26–50%; ++++, 51–75%; ++++, 76–95%; and ++++++, >95%.

Statistical Analysis. Synergy was tested using a two-sample, one-tailed, t test between two populations. The first population is the one with the mean that is equal to the average NO production when 4-HPR and IFN-γ (or TAM) are present. The second population is the one in which the mean is the sum of the average NO production from 4-HPR and the average NO production from IFN-γ (or TAM). The null hypothesis is that the means of the two populations are the same. The alternative hypothesis is that the mean of the first population is greater than that of the second. The null hypothesis (no synergy) is rejected in favor of the alternative hypothesis (synergy) if the P of the t test is less than 0.05.

Results

4-HPR-induced Inhibitory Effects Were Directly Correlated with NO Production. To assess the effect of 4-HPR on growth inhibition and NO production in breast cancer cells, ER+ (MCF-7 and T47D) and ER− (SKBr-3 and MDA-MB-453) cells were treated with 4-HPR (1, 2.5 μM). We selected 1 and 2.5 μM 4-HPR because these doses are clinically achievable (8). The ER status of each cell line was verified by Western blot analysis (Fig. 1). 4-HPR induced dose-dependent growth inhibition in all of the cell lines (Table 1A). 4-HPR also induced NO production in a dose-dependent manner in all of the cell lines (Table 1B). 4-HPR-mediated growth inhibition was directly correlated with NO generation. The ability of 4-HPR to induce growth inhibition and NO production was substantially greater in ER+ cells than in ER− cells. The 2.5-μM concentration of 4-HPR increased NO production by 24-fold in ER+ cells and 3.3-fold in ER− cells compared with untreated cells.

Because 4-HPR is known to exert its growth-inhibitory effects on breast cancer cells via apoptotic induction (45), we determined whether NO production was associated with 4-HPR-mediated apoptosis. 4-HPR treatment resulted in a dose-dependent increase in the percentage of apoptotic cells, as evidenced by the rise in the sub-G1 peak (Fig. 2). 4-HPR-mediated apoptosis was also directly correlated with NO production (Fig. 2).

Inhibition of NO Production Suppressed 4-HPR-induced Inhibition. To determine whether NO is involved in 4-HPR-induced inhibition, we used the NOS competitive inhibitor L-NMMA to inhibit NO production. T47D and MCF-7 cells were treated with 4-HPR (1 μM) in the presence and absence of L-NMMA. L-NMMA at the concentrations used (1, 10, 100 μM) was not cytotoxic to cells. L-NMMA effectively suppressed 4-HPR-induced NO production in a dose-dependent manner in both of the cell lines (Table 2). Inhibition of NO production prevented 4-HPR from inducing growth inhibition. L-NMMA at a concentration of 100 μM was able to return NO levels and cell counts to those of untreated breast cancer cells. These data indicate that NO induction is an essential mechanism by which 4-HPR induces growth inhibition.

Table 1

<table>
<thead>
<tr>
<th>4-HPR (μM)</th>
<th>MCF-7</th>
<th>T47D</th>
<th>MDA-MB-453</th>
<th>SKBr-3</th>
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<tr>
<td>0</td>
<td>264.0 ± 2.98</td>
<td>60.5 ± 2.24</td>
<td>64.5 ± 2.64</td>
<td>55.0 ± 2.37</td>
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<tr>
<td>1</td>
<td>28.0 ± 1.41</td>
<td>18.0 ± 0.50</td>
<td>40.0 ± 1.73</td>
<td>23.0 ± 0.87</td>
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<tr>
<td>2.5</td>
<td>7.3 ± 0.289</td>
<td>6.5 ± 0.50</td>
<td>20.0 ± 0.43</td>
<td>12.5 ± 0.43</td>
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A. Growth inhibition, total cell count (× 10⁶)*

B. NO production, μM nitrite/1 × 10⁶ cells**

Fig. 1. Expression of ERα in breast cancer cells. Western blot was performed to determine the levels of ERα in MCF-7, T47D, SKBr-3, and MDA-MB-453 breast cancer cells.

Fig. 2. 4-HPR-induced apoptosis was directly correlated with NO production. T47D cells were plated (1 × 10⁶ cells/well) in 6-well plates in DMEM/F12 medium supplemented with 5% FBS. Cells were treated with 4-HPR (1, 2.5 μM) for 5 days. Approximately 1 × 10⁶ cells were collected, and apoptosis was evaluated by flow cytometric analysis of propidium iodide staining. NO production was determined by measuring its stable end product nitrite using a Colorimetric Non-enzymatic Nitric Oxide Assay kit. *, the percentage of apoptotic cells (i.e., cells in the sub-G₁ peak); †, μM nitrite/10⁶ cells.
4-HPR Increased the Expression of NOSII and NOSIII in Breast Cancer Cells. Because NO is produced by NOS, we wanted to determine which isoform(s) was responsible for 4-HPR-induced NO production in breast cancer cells. T47D cells were treated with 4-HPR alone (1, 2.5 μM) and subjected to immunohistochemical staining for the different NOS isoforms (NOSII and NOSIII; Table 3). NOSII expression was not detected in untreated T47D cells, and fewer than 5% of cells exhibited a light NOSII staining. NOSII and NOSIII expression levels were increased in cells treated with 1 μM 4-HPR. A higher intensity of staining and a higher percentage of positive cells for NOSII and NOSIII were observed in cells treated with 1 μM 4-HPR in comparison with untreated cells (Fig. 3). NOSII had a higher intensity of staining than did NOSII in cells treated with 1 μM 4-HPR; however, a similar percentage of positive cells was observed (Table 3). 4-HPR at the 2.5-μM concentration showed dramatic increases in intensity and percentage of positive cells for NOSII. Although NOSII intensity was lower for the 2.5-μM concentration of 4-HPR than for the 1-μM concentration, a greater percentage of cells stained positive for NOSII expression at the 2.5-μM concentration than at the 1-μM concentration (Table 3). NOSII staining was higher in intensity and in percentage of cells than NOSII staining when cells were treated with 2.5 μM 4-HPR. Thus, 4-HPR mediates increases in NO production by inducing NOSII and NOSIII expression.

IFN-γ and TAM Enhanced 4-HPR-induced Apoptosis in Breast Cancer Cells. IFNs and TAM are known to potentiate retinoid-induced growth inhibition in breast cancer cells (12–14, 16, 46–48). To confirm the ability of IFNs and TAM to enhance 4-HPR-mediated growth inhibition, T47D cells were treated with 4-HPR alone or in combination with IFN-γ or TAM. At the 1-μM concentration, 4-HPR resulted in a slight increase in the percentage of apoptotic cells (Fig. 4). Alone, IFN-γ and TAM at the concentrations used had little effect on apoptosis, but they enhanced 4-HPR-mediated apoptotic activity. The percentage of apoptotic cells was increased from 7.3% to 27.7 and 14.7% when 4-HPR was combined with IFN-γ and TAM, respectively.

IFN-γ and TAM Enhanced 4-HPR-induced NO Production. Fig. 5 shows the effect exerted by 4-HPR in combination with IFN-γ on NO production. Production of NO was not affected by IFN-γ treatment alone in any of the cell lines. IFN-γ enhanced 4-HPR-mediated NO production in a dose-dependent manner in all of the cell lines, although ER− cells were more sensitive to this combination than were ER+ cells. There were 3.6-fold and 2.0-fold increases in NO production over that observed for 4-HPR alone with the 100-μM dose of 4-HPR in combination with 4-HPR in ER− and ER+ cells, respectively. A synergistic effect of IFN-γ and 4-HPR on NO production was observed in MCF-7 and T47D cells at all three doses of IFN-γ used (P < 0.05). In SKBr-3 cells, a synergistic effect on NO production was observed when the 50- and 100-μM doses of IFN-γ were used in combination with 4-HPR (P < 0.05). In MDA-MB-453 cells, an additive effect was observed when 4-HPR was combined with IFN-γ at a dose of 50 μM/μl, and a synergistic effect was observed when 4-HPR was combined with 100 μM/μl IFN-γ (P < 0.05).

TAM increased 4-HPR-induced NO production in ER− cells (Fig. 6). A 2.2-fold increase in NO production over that observed for 4-HPR alone was obtained with the 1-μM dose of TAM in combination with 4-HPR in ER− cells. TAM and 4-HPR had a synergistic effect on NO production in MCF-7 cells at both doses of TAM (P < 0.05). For T47D cells, a synergistic effect on NO production was observed when 1.0 μM TAM was combined with 1 μM 4-HPR (P < 0.05), whereas an additive effect was observed when 0.1 μM TAM was combined with 1 μM 4-HPR. The TAM and 4-HPR combination was not very effective in ER− cells. TAM did not enhance NO production in SKBr-3 cells or MDA-MB-453 cells, except when 4-HPR was combined with the highest dose of TAM.

4-HPR in Combination with IFN-γ or TAM Enhanced NOSII Expression. Table 3 illustrates the intensity and percentage of NOS immunostainings for cells treated with a combination of 4-HPR and IFN-γ or TAM. NOSII and NOSIII

<table>
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<th>Table 2</th>
<th>Inhibition of NO production suppressed 4-HPR-mediated growth inhibition in breast cancer cells</th>
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<tr>
<td></td>
<td>4-HPR (1 μM)</td>
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<tr>
<td>T47D cells</td>
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* NOS competitive inhibitor.

† Total cell counts were determined by trypan blue exclusion. Values are means ± SD of experiments performed in triplicate.

‡ Total NO production was determined by measuring its stable end product nitrite. Nitrite values were normalized to cell number. Values are means ± SD of experiments performed in triplicate.

<table>
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<th>Table 3</th>
<th>4-HPR increased NOS expression in breast cancer cells</th>
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<tr>
<td></td>
<td>Intensity*</td>
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<td></td>
<td>NOSII</td>
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<tr>
<td>Untreated</td>
<td>+</td>
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<tr>
<td>4-HPR (1 μM)</td>
<td>+</td>
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<tr>
<td>4-HPR (2.5 μM)</td>
<td>+++</td>
</tr>
<tr>
<td>IFN-γ (50 IU/ml)</td>
<td>–</td>
</tr>
<tr>
<td>IFN-γ + 4-HPR™</td>
<td>+++</td>
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<tr>
<td>TAM (1 μM)</td>
<td>–</td>
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<tr>
<td>TAM + 4-HPR™</td>
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* Intensity of NOS immunostainings: –, none; +, light; ++, moderate; ++++, intense.
† Percentage of NOS immunostainings: –, <5%; +, 5–25%; ++, 26–50%; ++++, 51–75%; ++++, 76–95%; +++++, >95%.
‡ 50 IU/ml IFN-γ and 1 μM 4-HPR were used.

1 μM TAM and 1 μM 4-HPR were used.
were not detected in cells treated with IFN-γ alone. NOSIII was also not detected in cells treated with TAM alone; however, both the intensity and percentage of NOSII immunostainings were increased in TAM-treated cells compared with untreated cells. Both IFN-γ and TAM, when combined with 1 μM 4-HPR, enhanced the expression of NOSII in comparison with 4-HPR alone (Fig. 7A). In contrast, NOSIII expression was not detected in cells treated with 4-HPR in combination with IFN-γ or TAM (Fig. 7B). The intensity and percentage of NOSII positive cells were greater for the 4-HPR and IFN-γ combination than for the 4-HPR and TAM combination, which was correlated with higher apoptotic rates (Fig. 4) and induction of NO production by the 4-HPR and IFN-γ combination (Figs. 5 and 6). Thus, IFN-γ and TAM enhance 4-HPR-mediated NO production by increasing NOSII expression.

Discussion

We have identified NO production as a novel mechanism by which 4-HPR induces apoptosis in breast cancer cells. The production of NO is specific to 4-HPR because the NOS inhibitor, L-NMMA, can selectively abrogate 4-HPR-induced inhibitory effects. Depending on the concentration of NO in the microenvironment, the effects of NO can be tumor promoting or tumor suppressing. Low concentrations of NO may protect some cell types from apoptosis induced by DNA-damaging agents (49) and increase the invasiveness and the metastatic potential of murine tumors (50, 51). But, high concentrations of NO are cytotoxic (52) and can prevent murine tumors from metastasizing (53, 54). Our findings that high concentrations of NO are inhibitory to breast cancer cells agree with previous findings (36–40).

We found that 4-HPR increases the expression of NOSII and NOSIII in breast cancer cells. NOS activity has been detected in human breast cancer (39, 55). The expression of NOSIII is restricted to the vascular endothelial cells within the breast tumors (56). NOSII is detectable in benign breast tumors (56) and breast hyperplasia (57). NOSII has been found predominantly in the stromal cells of breast tumors (55, 56, 58); however, some studies suggest that NOSII is expressed mainly in breast tumor cells (39, 59). The endogenous expression of NOSII within breast tumor cells has been inversely correlated to the tumor’s metastatic potential and may have an inhibitory role on breast cancer metastasis (56). On the other hand, high expression of NOSII within the...
stromal cells of breast tumors (55, 58) or within tumors $\geq 2$ cm (59) has been correlated with breast cancer metastasis, possibly because of its association with higher vascular density and increased angiogenesis (58). Our data indicate that 4HPR-induced high concentrations of NO by NOSII are inhibitory to breast cancer cells. The effects of 4HPR in breast stromal cells remain to be investigated.

We demonstrated that 4-HPR induced growth inhibition and NO production in both ER$^+$ and ER$^-$ breast cancer cells; however, ER$^-$ cells were less responsive than ER$^+$ cells to the effects of 4-HPR. A lower sensitivity of ER$^-$ cells to 4-HPR inhibitory effects in comparison with ER$^+$ cells is consistent with the results of previous studies (1). Our present results indicate that 4-HPR induces NO production by

...
increasing the expression of NOSII and NOSIII. Although NOSII expression has not been correlated with ER expression, the expression of NOSII has been correlated with the expression of progesterone receptors (39), which is an indicator of functional ER (60). NOSIII expression is found mainly in ER+/H11001 breast cancer cells (61, 62). Although only four cell lines were used, we speculate that a reduced expression of NOSII and NOSIII may be one potential explanation for the lower sensitivity of ER+/H11002 cells to 4-HPR. Future studies will be conducted to determine whether 4-HPR induces the transcriptional expression of NOS.

In the present study, the IFN-γ and 4-HPR combination resulted in additive or synergistic effects on NO production in all of the breast cancer cell lines investigated; however, ER− cells were less responsive to this combination. Furthermore, our results show that TAM enhanced 4-HPR-mediated NO production in ER+ but not in ER− breast cancer cells. The mechanisms by which IFN-γ or TAM enhance 4-HPR action are unknown. We have shown that one potential mechanism by which IFN-γ or TAM enhances 4-HPR-mediated growth inhibition is by increasing NO production via NOSII. A reduced ability to express NOSII may possibly be responsible for the lower sensitivity of ER− cells to 4-HPR. Future studies will be conducted to determine whether 4-HPR induces the transcriptional expression of NOS.

In the present study, the IFN-γ and 4-HPR combination resulted in additive or synergistic effects on NO production in all of the breast cancer cell lines investigated; however, ER− cells were less responsive to this combination. Furthermore, our results show that TAM enhanced 4-HPR-mediated NO production in ER+ but not in ER− breast cancer cells. The mechanisms by which IFN-γ or TAM enhance 4-HPR action are unknown. We have shown that one potential mechanism by which IFN-γ or TAM enhances 4-HPR-mediated growth inhibition is by increasing NO production via NOSII. A reduced ability to express NOSII may possibly be responsible for the lower response of ER− cells to the 4-HPR/IFN-γ and the 4-HPR/TAM combinations. We are aware that our results are in contrast to those of Coradini et al. (12), who reported that the effectiveness of the 4-HPR/TAM and 4-HPR/IFN combinations was independent of ER status. These discrepancies may be because we used different ER− cell lines (MDA-MB-453 and SKBr-3 versus BT20 and MDA-MB-231) and different IFN (type II, γ versus type I, β) than they did. 4-HPR has potential chemopreventative effects in breast cancer (64, 65), and oral leukoplakia (66, 67). It remains to be determined whether the ability of 4-HPR to induce apoptosis via NO production is a mechanism specific to breast cancer cells or whether it represents a common apoptotic pathway in other cancer types as well. Nonetheless, furthering our understanding of the molecular mechanisms of 4-HPR action alone and in combination therapies will benefit the clinical application of 4-HPR in all of these cancers.

To summarize, we demonstrated a critical role of NOS induction and NO production in 4-HPR-mediated inhibition in breast cancer cells. Pharmacologically achievable doses of 4-HPR and TAM (68) were used in the present study. Thus, we believe our present study is highly relevant to the ongoing clinical trials. We speculate that determining the level of ER as well as NOS expression in breast tumor cells may allow us to identify patients who will most likely respond to 4-HPR.

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References

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Minireview

Synopsis of a Research Roundtable Presented on Cell Signaling in Myeloma: Regulation of Growth and Apoptosis—Opportunities for New Drug Discovery

Kenneth C. Anderson and William S. Dalton

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Abstract

A wide variety of alterations affect important cell-signaling pathways involved in growth, survival, and migration of myeloma cells. Several of these pathways have been identified, and a number of potential anticancer agents are in development to block specific cell signaling proteins. The fifth experts’ roundtable in multiple myeloma was convened in May 2001 to focus on this important issue. The roundtable brought together myeloma experts, researchers involved in cell signaling, industry scientists, and investigators studying other hematologic malignancies, with the single purpose of challenging current thought and increasing the collective knowledge in the evolving field of cell signaling and multiple myeloma. The session was cochaired by Dr. William S. Dalton of the H. Lee Moffitt Cancer Center and Dr. Kenneth C. Anderson of the Dana-Farber Cancer Institute. Sponsored by the Dana-Farber Cancer Institute, the roundtable was funded by the Multiple Myeloma Research Foundation and McCarty Cancer Foundation of Canada.

Introduction

MM is a plasma cell malignancy characterized by migration and localization to the bone marrow from where cells disseminate and facilitate formation of bone lesions. Despite all available therapies, MM is generally considered incurable and will affect ~14,000 individuals in the United States in 2002 (1, 2). MM typically displays a low proliferative rate and ultimately develops resistance to currently available therapeu tic agents. Apoptosis is the primary means by which most chemotherapy and radiotherapy modalities kill cancer cells (3). This cytotoxic response uses signal transduction pathways common in physiological mechanisms of programmed cell death or apoptosis. A block in programmed cell death is believed to be a major contributing factor to the drug resistance observed in MM. To prevent apoptosis, tumor cells acquire mechanisms to prevent cell death. Some of these mechanisms are mediated by extracellular growth and survival factors and the tumor microenvironment (4, 5).

Tumor progression is a multistep process determined by events intrinsic to the tumor cell and microenvironment. Environmental factors that contribute to the pathogenesis and progression of human malignancies include soluble factors, such as cytokines, hormones, and growth factors; interaction of the tumor with extracellular matrix molecules; and interactions of the tumor with healthy extracellular cells and tissues. The presence of growth factors, such as IL-6, the most important growth factor in MM, may block cell death associated with chemotherapies and radiotherapies (3, 5).

Growth Control, Survival, and Apoptosis

Translocations and Mutations. Although for most normal B-cell differentiation and for many B-cell malignancies critical signals are transduced by the B-cell receptor, this is probably not the case for myeloma, a plasma cell malignancy. It is most likely that in the terminal stages of plasma cell differentiation, other signals coming from the microenvironment are more important mediators of growth and survival. Like their normal counterpart, myeloma cells are naturally long lived, and presumably genetic mutations are not required to increase this life span. In keeping with this, the translocations and point mutations that have been identified primarily appear to promote growth, rather than survival. The key oncogenes involved are cyclin D1, cyclin D3, FGFR3, c-maf, mafB, and c-myc (6, 7).

Primary translocations are mediated by switch combinations and, to a lesser extent, somatic hypermutations. Translocations mediated by these mechanisms occur in 75% of patients with MM. With disease progression, secondary translocations that result from general chromosomal abnormalities are observed. In contrast to primary translocations, secondary translocations can be very complex. Recent re-
search highlights the important role of FGFR3 in primary translocations and c-myc in secondary translocations in myeloma. FGFR3 is translocated in ~25% of myeloma cell lines and ~20% of primary tumors. Overexpression of FGFR3 in a murine bone marrow transplant model results in enhanced cellular proliferation in response to IL-6 and complete independence from IL-6 in some situations (8). The reasons for enhanced cellular proliferation are still unclear; however, up-regulation of STAT3 and Bcl-xL appears to explain the reduced apoptosis that is observed. Thus, FGFR3, STAT3, and Bcl-xL are all potential targets for myeloma therapy. An FGFR inhibitor (SU5402) developed by SUGEN, Inc. inhibits growth of MM lines in vitro in an FGFR3-specific fashion and represents a potential therapy for myelomas bearing this translocation.

**IL-6 and STAT Activation.** Most conventional chemotherapies for MM exert their effects, at least in part, via disruption of the IL-6-dependent cell growth pathway. In MM, the interaction between tumor and BMSCs triggers production of cytokines, such as IL-6 (5). Elevated IL-6 levels observed in patients with MM contribute to chemo-resistance and treatment failure by mechanisms that increase tumor growth and survival (9). Moreover, IL-6 inhibits dexamethasone-induced tumor apoptosis (5). Dexamethasone-induced apoptosis in MM is associated with down-regulation of growth-related signaling pathways, such as MAPK and p70rsk. MAPK is a relatively late event during IL-6 signaling, and more upstream gp130-associated kinases, such as members of the Janus family of kinases (JAK1, JAK2, JAK3) and the STAT family of proteins (STAT1, STAT3), may be important targets (4).

STAT3 mediates IL-6 activities. When IL-6 is engaged by the high-affinity receptor, it activates STAT3. Receptors of both gp80 and gp130 are expressed when cells are in the activated B-cell stage. When induced by IL-6, these cells become plasma cells, and both gp80 and gp130 are shut off. Therefore, IL-6, by way of activating STAT and downstream events, actually down-regulates both receptors. This is the first evidence of negative regulation of gp130 in any system. This implies that IL-6 negatively controls its own receptor expression. In contrast, there is no down-regulation in myeloma cells, which exhibit continued proliferation and STAT3 activation in response to IL-6, possibly attributable to up-regulation of the IL-6 receptor (10, 11). Therefore, IL-6 signaling is different in B cells as compared with malignant cells, and the difference is in receptor regulation. The challenge is to determine which point downstream of IL-6 leads to negative regulation of gp130.

Although STATs are activated by numerous physiological signals, their constitutive expression in a wide variety of human cancer cells is linked to persistent activation of tyrosine kinases leading to continued STAT signaling and cell proliferation (12). In a number of tumor models, inactivation of STATs is associated with a loss of survival or cell proliferation. Because normal cells are fairly tolerant of inhibition of STAT signal transduction, STATs are an attractive target for cancer therapy. STAT activation is an important event in endothelial cell proliferation and may contribute to angiogenesis, a critical process in tumor growth (13). Therefore, modulation of STAT function may be important not only in directly inhibiting tumor growth but also as an antiangiogenic strategy.

**Cell Adhesion and Survival.** It is possible that cell adhesion creates an antiapoptotic environment that allows for the progression of hematopoietic tumors, particularly myeloma (14). Although intrinsic factors like overexpression of constitutively active receptors may be important, extrinsic factors such as the microenvironment may also participate in the pathogenesis of MM. In addition to soluble factors like IL-6, there are other means by which the environment can influence cancer cells, e.g., through direct cell contact. A number of cell adhesion molecules are involved in cell signaling, and some of these signaling events can influence cell survival. When myeloma cells adhere to fibronectin, they become resistant to chemotherapeutic agents, such as doxorubicin and melphan, as well as radiation therapy. The protective effects of adhesion to fibronectin can be avoided if β1 integrins are blocked (15).

FAS-induced apoptosis is also inhibited by β1 integrins, and inhibition occurs at a very proximal event demonstrated by a blocking of procaspase 8 activation. A number of inhibitors can block FAS-induced apoptosis. One of these inhibitors is c-FLIPc. Theoretically, the more FLIPc that is present, the more resistance there will be to FAS-induced apoptosis attributable to inhibition of procaspase 8. When myeloma cells are adhered to fibronectin, there is a dramatic increase in cytoplasmic FLIPc concentration (3). In contrast, virtually no FLIPc is detected when cells are in suspension. Therefore, by adhering to fibronectin, myeloma cells are protected from FAS-induced apoptosis. This may allow myeloma cells to escape immune surveillance and contribute to myeloma progression (3).

**Novel Treatment Approaches**

Despite the availability of a growing number of biological, chemotherapeutic, and combination regimens, the prognosis of MM remains poor. Therefore, numerous researchers are investigating novel approaches to treatment.

**Proteasome Inhibitors.** Proteasome inhibitors represent novel anticancer agents for the treatment of MM because of their effects on multiple pathways in the pathogenesis of the disease. These include regulation of cell cycle progression by blocking degradation of ubiquinated cyclins and cyclin-dependent kinase inhibitors, induction of tumor apoptosis,

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and suppression of gene transcription by preventing activation of NF-κB (5). The effects of the proteasome inhibitor PS-341 on myeloma proliferation were evaluated using myeloma chemosensitive and chemoresistant cell lines and human myeloma cells (16). PS-341 suppressed tumor cell proliferation and induced apoptosis, even in myeloma cells resistant to chemotherapy and dexamethasone. In fact, PS-341 overcame IL-6-induced protection against dexamethasone-induced apoptosis and suppressed IL-6-induced growth through inhibition of MAPK. PS-341 also suppressed paracrine tumor growth by blocking NF-κB-dependent IL-6 secretion from BMSCs and decreasing myeloma cell adherence to BMSCs (5). Remarkably, PS-341 with or without chemotherapy had no effect on normal bone marrow and blood leukocytes. Thus, PS-341 reduces NF-κB activity in myeloma and is cytotoxic to myeloma cells without an affect on normal cells.8

Inhibitors of Angiogenesis. Angiogenesis is essential for the sustained growth of solid tumors (17). Tumors that lack adequate vasculature do not grow beyond a limited size. However, tumors that undergo neovascularization acquire the ability to grow rapidly and generally exhibit increased metastatic potential. Bone marrow angiogenesis is critical for the progression of several leukemias. Angiogenesis is accomplished by the enhanced production and release of VEGF that occurs during contact between myeloma and stromal cells (18). Coculture studies indicate stromal cell-derived IL-6 is the major stimulus for VEGF, which in turn amplifies the response by autocrine stimulation of IL-6 production. Thus, angiogenesis inhibitors that target VEGF or the VEGF receptor have the potential to inhibit myeloma in two ways. They can: (a) inhibit angiogenesis; or (b) block the ability of VEGF to stimulate vascular endothelial cells to produce IL-6. SU5416 and SU6668 are novel small molecule antiangiogenic agents that inhibit VEGF signaling through receptor tyrosine kinases essential to angiogenesis, including the VEGF receptor Flk-1/KDR (19, 20). Both agents have been evaluated for antitumor activity in preclinical models as single agents and in combination with standard cytotoxic agents and radiation. SU5416 is a selective inhibitor of Flk-1/KDR. In a Phase II trial of SU5416 in patients with acute myelogenous leukemia, there was an overall response rate of 30% by day 28 of therapy. Phase II trials in patients with MM are currently ongoing.9

SU6668, which is structurally related to SU5416, is currently in Phase I trials. SU6668 also inhibits Flk-1/KDR, although its activity against this receptor is 10-fold less than that of SU5416. The more broadly acting SU6668 reduced tumor vascularity, which was accompanied by increased tumor cell death, decreased proliferation, and a concomitant rise in VEGF gene transcription in mice (21). In the A431 human epidermoid tumor model, p.o. therapy with SU6668 completely regressed tumors that had grown to between 500 and 2000 mm3. When therapy was stopped, tumors remained regressed in the majority of the mice. In the few animals in which tumors regrew, reinitiation of therapy with SU6668 regressed tumors a second time with no evidence of resistance. SU6668 is about to enter Phase I studies against a variety of hematologic malignancies, including MM.8

Antisense Strategies. Apoptosis involves two pathways: (a) one that is mitochondria independent; and (b) one that is mitochondria dependent. The mitochondria-dependent pathway is involved in chemotherapy-induced apoptosis. To overcome mitochondrial-dependent resistance, an antisense oligonucleotide can be used to turn down the Bcl-2 protein. Preclinical and clinical studies suggest that oblimersen sodium, a Bcl-2 antisense oligonucleotide (G3139; Genta) that down-regulates Bcl-2 in human tumors, synergizes with cytotoxic and immunotherapeutic agents against various hematologic malignancies and tumors (22). Results from a Phase I study using oblimersen sodium were published last year. Patients in the study had low-grade malignancy and were heavily pretreated.10 One patient had a complete response and has retained this response for 4 years. There was disease stabilization in eight other patients. In the patients who displayed a response to therapy, a significant down-regulation of Bcl-2 in bone marrow, lymphocytes, and lymph node samples was documented. In seven of eight responders, down-regulation of the Bcl-2 protein was also documented. Reduction in platelet count was the dose-limiting toxicity, which occurred at approximately the same dose associated with unacceptable toxicity with other antisense oligonucleotides. The toxicity is reversible in 4–5 days after discontinuation of treatment. This dose is much higher than the dose required to reduce B cells to the therapeutic level required. The results of this study confirmed that down-regulation of a protein through an antisense approach is clinically viable.10 Phase III studies were initiated in 2001 for advanced MM at 65 centers in the United States, Canada, and Great Britain (23).

Fras and Geranylgeranyl-Transferase I Inhibitors. The fact that proteins such as Ras, Rac, and Rho A require farnesylation or geranylgeranylation for inducing malignant transformation prompted many investigators to develop FTIs and geranylgeranyl-transferase I inhibitors as novel anticancer drugs (24). FTIs antagonize oncogenic signaling, reverse malignant transformation, inhibit human tumor growth in nude mice, and induce tumor regression in transgenic mice without toxicity. One study evaluated the effects of FTIs in patients with MM, non-small cell lung cancer, and colorectal cancer. Preliminary biochemical data from four patient samples demonstrated that ex vivo exposure to the FTI inhibited FTase activity. Therefore, at clinically relevant doses, FTIs are capable of blocking farnesylation of critical proteins in-


involved in myeloma progression. A Phase II clinical trial is currently ongoing in myeloma.\textsuperscript{11}

**Purine Analogues.** The purine analogue 8-Cl-Ado demonstrates cytotoxicity against MM both *in vitro* and *in vivo* (25). It mediates programmed cell death against MM cell lines resistant to traditional chemotherapeutic agents.\textsuperscript{12} 8-Cl-Ado induces formation of a classical chromatin ladder, poly (ADP-ribose) polymerase cleavage, accumulation of cells in the sub-G<sub>1</sub> cell cycle fraction, increased activity of caspases 8 and 9, and a decrease in mitochondrial membrane potential. This compound is effective against a broad spectrum of both solid tumors and hematologic malignancies. Although it kills normal lymphocytes *in vitro*, it produces very little hematopoietic toxicity in animal models. 8-Cl-Ado is phosphorylated through the adenosine kinase pathway to the monophosphate and ultimately to the triphosphate derivative (8-chloro ATP), which is thought to be the cytotoxic metabolite (25). In myeloma cells, ~40% is converted to the monophosphate derivative, ~40% into the triphosphate, and the remainder to the diphosphate.\textsuperscript{12} Adenosine kinase is required for cell killing. The primary effects of 8-Cl-Ado are on mRNA synthesis (50% inhibition) and to a lesser degree on RNase. There is no effect on tRNA. In contrast to other purine analogs, 8-Cl-Ado does not affect DNA synthesis. Eight-Cl-Ado may be an effective agent in drug-resistant myeloma.

**Tyrosine Kinase Inhibitors.** Tyrosine kinases represent a family of enzymes that are critical for cell growth, proliferation, and survival (19). These include FGFRs and VEGFRs, both of which are considered central to local autocrine and paracrine mechanisms that support and perpetuate myeloma growth and proliferation. FGFR3 is a major target for tyrosine kinase inhibition, because it is overexpressed in MM patients having the t(4;14) gene translocation (19). Small molecule inhibitors of enzyme targets are being investigated in programs directed against, among others, tyrosine kinases known or thought to be important in cell signaling in transformed cells.\textsuperscript{13} Certain pyrido [2,3-d] pyrimidines are potent selective inhibitors of c-Src kinases and FGFRs, whereas quinazolines primarily block VEGFRs and FGFRs (19, 26). The transforming tyrosine kinase Bcr-Abl is considered an ideal target to validate the clinical utility of protein kinase inhibitors as therapeutic agents in chronic myelogenous leukemia that may be applicable to MM (27, 28). These observations suggest that small molecule inhibitors of signal transducing transforming proteins may have clinical potential and represent effective and novel approaches to treatment of neoplastic disease, including MM.\textsuperscript{13}

**Recommendations for Future Research**

Cell growth and apoptosis are essential, yet opposing, cellular processes. Cross-talk between these signaling pathways regulates the growth and survival of tumor cells. Characterization of the mechanisms regulating myeloma growth and resistance to apoptosis *in vitro* provides a framework for understanding potential ways to specifically inhibit cell growth and/or trigger apoptosis.\textsuperscript{3} An understanding of the apoptotic pathways is instrumental to the development of new therapies that will enable us to pinpoint where to attack tumor cells.\textsuperscript{14} Defining the signaling pathways mediating growth and survival, as well as angiogenesis, may help derive new and effective treatment strategies in MM.\textsuperscript{3} Approaches to blocking signal transduction pathways mediated by IL-6 and other cytokines may enhance cytotoxic drug activity (3).

In addition to signaling pathways, further research is needed to identify ways that the microenvironment, including cell adhesion, influences how cells respond to immune surveillance. Interactions between tumor cells and environmental factors may help to explain not only the pathogenesis of malignant disease but also the protective mechanisms contributing to the selection and outgrowth of drug-resistant tumors (3).

Studies of signal transduction will lead the way into the next generation of anticancer agents. It is unlikely that new alkylating agents or new anthracyclines will have a huge impact on myeloma therapy. We need to further investigate what goes on in the normal cell and understand how it differs from a malignant cell to develop a new generation of anticancer compounds.\textsuperscript{7} Further definition of the heterologous network of growth, survival, and apoptotic factors may provide clues to the development of better treatments of MM and other myeloid malignancies.\textsuperscript{15}

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References


Erratum
In the article by Simeone et al., entitled “A Novel Mechanism by Which N-(4-hydroxyphenyl)reti-namide Inhibits Breast Cancer Cell Growth: The Production of Nitric Oxide,” which appeared in the October 2002 issue of MCT (pp. 1009–1017), Figure 7 appeared in black and white but should have appeared in color as seen below. The authors do, however, possess reprints that include the correct color version of the figure.

Fig. 7. NOSII expression was increased when 4-HPR was combined with IFN-γ or TAM. T47D cells were plated (1 x 10^5 cells/well) in 6-well plates in DMEM/F12 medium supplemented with 5% FBS. Cells were treated with 4-HPR (1 μM) alone or in combination with IFN-γ (50 IU/ml) or TAM (1 μM). Cells treated with 4-HPR alone or in combination with TAM were incubated for 5 days, and cells treated with a combination of 4-HPR and IFN-γ were incubated for 4 days. Cells were harvested and immunostained for NOS isoforms. A, NOSII immunostainings; B, NOSIII immunostainings. Untreated (i); 1 μM 4-HPR (ii); IFN-γ (iii); IFN-γ and 4-HPR (iv); TAM (v); TAM and 4-HPR (vi).
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