Experimental Studies on the Effects of the Combined Use of N-(4-hydroxyphenyl)retinamide (4-HPR) and Tamoxifen (TAM) for Estrogen Receptor (ER)-Negative Breast Cancer

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Summary: We investigated the effects of combination therapy with N-(4-hydroxyphenyl)retinamide (4-HPR) and tamoxifen (TAM) on estrogen receptor (ER) negative breast cancer, for which no effective supplementary therapy has been established, using the human breast cancer cell line MDA-MB-231. TAM or 4-HPR alone had little antitumor effect, but the combined use of TAM and 4-HPR had a strong cell growth inhibitory effect. Cell cycle analysis by flow cytometry showed an increased frequency of the G2/M phases in the 4-HPR-TAM combination group. Measurement of $^3$H-TAM incorporation into the cell showed that, compared with the TAM group, the 4-HPR-TAM combination group incorporated about 1.45 times more TAM into the cell. Thin-layer chromatographic analysis of changes in the cell membrane ganglioside GM$_3$ showed a marked increase in GM$_3$ in the 4-HPR-TAM combination group. We speculate that the administration of TAM in the presence of 4-HPR changes the membrane glycolipid GM$_3$, increasing intracellular TAM concentrations, thus exerting antitumor activity. Presumably, during this process, antitumor effects do not induce cell death but arrest the cell cycle in the G2 phase. Thus, the combined use of TAM and 4-HPR inhibited the growth of the ER-negative breast cancer cell line MDA-MB-231. These results suggest that combination therapy with TAM and 4-HPR can be a potent supplementary therapy also for ER-negative patients in clinical practice.

Key words: estrogen receptor negative breast cancer, N-(4-hydroxyphenyl)retinamide, tamoxifen, G2 arrest

INTRODUCTION

Although the usefulness of tamoxifen (TAM) in the treatment of estrogen receptor (ER) positive breast cancer has been well documented [1,2], no effective supplementary therapy for ER-negative breast cancer has been established; thus, the advent of potent supplementary therapy is eagerly awaited. Currently, in addition to TAM, promising preventive agents for breast cancer recurrence include an aromatase inhibitor [3], luteinizing hormone-releasing hormone agonists [4], monoterpenes [5], isoflavones [6], rexinoids [7], vitamin D [8], a tyrosine kinase inhibitor [9], a cyclooxygenase-2 inhibitor [10], and retinoids. Of these, N-(4-hydroxyphenyl)retinamide (4-HPR), a kind of retinoid, is a synthetic derivative of vitamin A, which accumulates selectively in the mammary glands and fat in the body and, compared with other synthetic retinoids, is less likely to cause hepatic damage. Thus, treatment with 4-HPR has attracted attention as a supplementary therapy for breast cancer which requires prolonged drug administration [11-13]. The reported mechanisms involved in the antitumor effects of 4-HPR on malignant tumors are that it decreases serum IGF-I levels [14], controls the cell cycle [15], inhibits aromatase activity [16], and...
induces apoptosis [17-19]. However, many of the mechanisms remain to be elucidated. To enhance its antitumor effects, retinoid in combination with hormone therapy, chiefly with TAM, has been tried. A study reported that the combination of retinoid administration and ovariectomy enhanced the tumor growth inhibitory effect [20]. In addition, there are scattered reports of basic studies on the effects of the combined use of retinoid and TAM [21], and phase I trials of combination therapy with retinoid and TAM have already been performed [22,23]. However, the mechanisms of the enhancement of antitumor effects by the combined use of retinoid and TAM remain unknown; thus, their elucidation is likely to lead to a new therapy for not only ER-positive but also ER-negative breast cancer. In this study, we investigated the effects of combination therapy with 4-HPR and TAM for ER-negative breast cancer for which no effective supplementary therapy has been established, using the human breast cancer cell line MDA-MB-231.

MATERIALS AND METHODS

Cell culture

The ER-negative and -positive breast cancer cell lines MDA-MB-231 and MCF7, respectively, were obtained from American Type Culture Collection (Rockville, MD). Both cell-lines were cultured in DMEM medium supplemented with 10% FBS at 37 ºC in an atmosphere of 5% CO2. The 4-HPR obtained from BIOMOL (Plymouth Meeting, PA) was dissolved in ethanol for use. TAM was obtained from WAKO. 1 x 10⁶ cells/well were seeded in 6-well culture plates and, 24 hs later, 0.5 µM 4-HPR alone, 1 µM TAM alone, or both were added to prepare 3 groups of cell cultures. After 120 hs, cells were suspended by treatment with 0.05% trypsin, and cells in each group were stained with trypan blue for cell counting.

Flow cytometry

Three groups of cell cultures were prepared by adding only 0.5 µM 4-HPR, only 1 µM TAM, or both. Cells were harvested 120 hs later, fixed with 70% ethanol, stained with propidium iodide (1 mg/ml), and analyzed for changes in the cell cycle by flow cytometry.

Measurement of TAM incorporation using ³H-TAM

Cultures of ER-negative MDA-MB-231 cells were divided into 2 groups according to the drugs added: the TAM group (1 µCi ³H-TAM) and the 4-HPR-TAM combination group (0.5 µM 4-HPR + 1 µCi ³H-TAM). Forty-eight hours after addition of the drug(s), the cells were washed, dissolved with triton X, and subjected to measurement with a scintillation counter. Similar experiments were performed using the ER-positive cell line MCF7.

Detection of ganglioside GM₃ by thin-layer chromatography

Cells were sonicated and re-sonicated after adding a 2:1 mixture of chloroform (C) and methanol (M). The supernatant was collected and evaporated to dryness with N₂ gas to extract the total lipid. This lipid was dissolved in C/M, the solution was centrifuged, and the supernatant was removed. C/M/water was added to the remaining lower layer and, after centrifugation, the upper layer was collected. This layer was evaporated to dryness with N₂ gas to yield a hydrophilic glycolipid and part of phospholipid. The hydrophilic lipid obtained was desalted on an equilibrated C18 Bond Elute column. Samples to be separated were prepared by dissolving the extracted glycolipid in isopropanol/hexan/water. Commercially available GM₃ (Diatron) was used as a control. Color was developed by orcinol-H₂SO₄. Samples were applied to the lower end of a HPTLC plate that had been dried at 100 ºC for 10 min, and were separated for about 60 min in a separation tank to which C/M/0.5% CaCl₂-H₂O had been added. Subsequently, the plate was dried with cool air, and color was developed.

Statistical analysis

Statistical analysis of p-values was made using the Student’s t-test. A level of p<0.05 was accepted as statistically significant.

RESULTS

Morphologic changes in breast cancer cells after the addition of TAM and 4-HPR, singly or in combination, and the antitumor effects

Figure 1a shows a photomicrograph of the microscopic appearance of cells at 72 hs after drug administration. Cells in the TAM or 4-HPR group did not significantly differ from those in the control group. In contrast, in the 4-HPR-TAM combination group, a marked decrease in the number of viable cells and the presence of numerous suspended cells were
COMBINED USE OF 4-HPR AND TAM FOR BREAST CANCER

**Fig. 1.** Morphologic changes in breast cancer cells after the addition of TAM and 4-HPR, singly or in combination, and the antitumor effects. (a) Cells in the TAM or 4-HPR group did not significantly differ from those in the control group. In contrast, in the 4-HPR-TAM combination group, a marked decrease in the number of viable cells and the presence of numerous suspended cells were observed, and the shape of the cells was changed from dendritic to round.

**Figure 1b** shows the number of viable cells at 120 hs after drug administration in the TAM, 4-HPR, and 4-HPR-TAM combination groups. The TAM and 4-HPR groups did not differ in cell growth from the control group. In contrast, 42% growth suppression was observed in the 1 μM TAM-0.5 μM 4-HPR combination group, and 56% growth suppression in the 5 μM TAM-0.5 μM 4-HPR combination group, indicating significant antitumor effects in the combination groups (p=0.003).

**Changes of cell cycle after the administration of TAM and 4-HPR singly or in combination**

Flow cytometry showed no significant difference in the cell cycle between the TAM and control groups. The frequency of the G2/M phases in the 4-HPR group was 20%, which was more frequent than 15% in the control group. The frequency of the G2/M phases in the 4-HPR-TAM combination group was...
Fig. 2. Changes of cell cycle after the administration of TAM and 4-HPR singly or in combination. Cells were harvested 120 hs later, fixed with 70% ethanol, stained with propidium iodide (1 mg/ml), and analyzed for changes in the cell cycle by flow cytometry. Similar results were observed in two additional experiments.

27%, which was clearly higher than that in the 4-HPR group (Fig. 2).

**Promotion of TAM incorporation by 4-HPR**

Compared with the TAM group, the 4-HPR-TAM combination group showed an approximately 1.45-fold increase in the incorporation of TAM into the cell (Fig. 3). In a similar experiment with the control ER-positive MCF7 cells, the TAM group already showed a high level of TAM incorporation, and the concomitant use of 4-HPR did not promote TAM incorporation (data not shown).

**Detection of changes in ganglioside GM3 by thin-layer chromatography**

The 4-HPR group did not undergo changes in cell membrane glycolipid. However, the TAM group showed a mild increase in GM3, and the 4-HPR-TAM combination group showed a marked increase in GM3 (Fig. 4).

Fig. 3. Promotion of TAM incorporation by 4-HPR. Cultures of ER-negative MDA-MB-231 cells were divided into 2 groups according to the drugs added: the TAM group (1 μCi ³H-TAM) and the 4-HPR-TAM combination group (0.5 μM 4-HPR+1 μCi ³H-TAM). Forty-eight hours after addition of the drug(s), the cells were washed, dissolved with triton X, and subjected to measurement with a scintillation counter.
DISCUSSION

TAM or 4-HPR alone had little antitumor effect on the ER-negative breast cancer cell line MDA-MB-231. However, TAM and 4-HPR in combination clearly exerted antitumor effects. In regard to the mechanisms of this antitumor activity, we speculated from these data that the changes in the membrane glycolipid GM₃ caused by the administration of TAM in the presence of 4-HPR and the increase in intracellular TAM levels caused changes in the cell cycle, thus producing antitumor activity. GM₃ has been reported to be highly expressed in breast cancer cells from patients with breast cancer [24]. Nohara et al. [25] have reported that GM₃ is highly expressed in MDA-MB-231 breast cancer cells, and that GM₃ may control the functions of growth factors. In this study, the expression of GM₃ was increased only in the 4-HPR-TAM combination group; moreover, suppression of cell growth was observed only in this group. These findings suggest that one of the causes of antitumor effects is the direct suppression of cell growth by GM₃, and that GM₃ may control the functions of growth factors. In this study, the expression of GM₃ was increased only in the 4-HPR-TAM combination group; moreover, suppression of cell growth was observed only in this group. These findings suggest that one of the causes of antitumor effects is the direct suppression of cell growth by GM₃, and that GM₃ may control the functions of growth factors.

Flow-cytometric analysis of the cell cycle suggested that the direct suppression of cell growth by TAM in the presence of 4-HPR was due to arrest of the cell cycle in the G2/M phases. Dipietrantonio et al. [28] have reported that the mechanism of G2 arrest of HL-60 cells by 4-HPR involves down-regulation of p34cdc2. Panigone et al. [29] have explained that 4-HPR causes G2 arrest by down-regulating cyclin D1 and thus inhibiting the phosphorylation of retinoblastoma protein (Rb). Also, it has been reported that the combined use of epirubicin and TAM arrests Ehrlich's carcinoma ascitic cells (EATC) in the G2 phase [30]. Thus, we speculate that 4-HPR or TAM exerts antitumor activity by somehow controlling the cell cycle. The results in this study suggest that the combined use of TAM and 4-HPR changes the expression of GM₃, which directly or indirectly changes the cell cycle, or that TAM itself, which has become easy to incorporate into the cell, influences the cell cycle. However, detailed investigation will be needed in the future to elucidate the mechanisms of G2 arrest of the cell cycle.

Furthermore, 4-HPR is known to induce apoptosis in sensitivity) by changing the expression of GM₃ in MDA-MB-231 cells. However, prior to this study, no reports have described the relationship of retinoid or TAM with ganglioside.

Fig. 4. Detection of changes in ganglioside GM₃ by thin-layer chromatography. Ganglioside GM₃ was detected by thin-layer chromatography as described under 'MATERIALS AND METHODS'.

<table>
<thead>
<tr>
<th>1μM TAM</th>
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<tr>
<td>0.5μM 4-HPR</td>
<td>(-)</td>
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positive control

[38.1] [36.5] [54.6] [60.6]
various malignant tumor cells. We also tried to detect apoptosis, but did not observe it by the TUNEL method (data not shown) or by flow cytometry. Therefore, we speculate that the antitumor effects of the combined use of TAM and 4-HPR on breast cancer cells involve arrest of the cell cycle, but not cell death.

In conclusion, the combined use of TAM and 4-HPR suppressed the growth of the ER-negative breast cancer cell line MDA-MB-231. This result suggests that combination therapy with TAM and 4-HPR can be a potent supplementary therapy also for ER-negative patients in clinical practice.

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REFERENCES


