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Antitumor Effect of 22-Oxa-calcitriol, a Noncalcemic Analogue of Calcitriol, in Athymic Mice Implanted with Human Breast Carcinoma and Its Synergism with Tamoxifen[1]

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ABSTRACT

The antitumor effect of 22-oxa-calcitriol (OCT), a newly developed noncalcemic analogue of calcitriol, was examined *in vivo* in athymic mice implanted with human breast carcinoma with or without estrogen receptor (ER). In ER-positive MCF-7 tumor, the growth of which was dependent on exogenous estrogen, administration p.o. of OCT as well as the antiestrogen tamoxifen five times a week for 4 weeks suppressed tumor growth in a dose-related fashion. The antitumor effect of 1.0 μg/kg body weight (BW) OCT (mean ± SEM of tumor weight in 6 mice: 28 ± 4% of vehicle-treated group) was comparable to that of 2.0 mg/kg BW tamoxifen (25 ± 6% of control group). In addition, a synergistic antitumor effect of submaximal doses of OCT and tamoxifen was observed in MCF-7 tumor *in vivo* as well as in ER-positive breast carcinoma cell lines (MCF-7 and ZR-75-1) *in vitro*. Administration of OCT p.o. three times a week for 4 weeks also suppressed the growth of ER-negative MX-1 tumor in a dose-dependent manner without raising serum calcium concentrations. The antitumor effect of 1.0 μg/kg BW OCT (mean ± SEM of tumor weight in 10 mice: 44 ± 6% of vehicle-treated group) was greater than that of 500 μg/kg BW Adriamycin (71 ± 6% of control group). These results indicate that OCT suppresses the growth of ER-negative as well as ER-positive breast carcinoma *in vivo* without causing hypercalcemia and that the antitumor effect of OCT can be enhanced by tamoxifen in an ER-positive tumor. It is suggested that OCT may provide a new strategy, either alone or in combination with other anticancer drugs, for systemic adjuvant therapy of breast carcinoma regardless of ER status.

INTRODUCTION

Breast carcinoma is one of the most common malignancies of women in many countries including Japan, where its management has been a major medical problem. Although much improvement has been made in the early detection of breast tumor and surgical treatment, endocrine therapy and/or cytotoxic chemotherapies still remain important as an adjuvant to surgical mastectomy and for patients with advanced disease (1).

$1,25(OH)_2D_3$[4] plays an important role along with parathyroid hormone in regulating calcium homeostasis and bone metabolism (2). An intracellular receptor molecule for $1,25(OH)_2D_3$ or vitamin D receptor has been identified, and its primary structure has been determined by molecular cloning, which has demonstrated that VDR belongs to the steroid and thyroid hormone receptor superfamily (3). VDR is widely expressed not only in its classic target organs involved in calcium metabolism, such as intestine, bone, and kidney, but also in a number of normal and malignant tissues (2). Since the first demonstration that VDR is present in breast cancer cells (4), it has been reported that $1,25(OH)_2D_3$ inhibits the growth of a variety of malignant cells, including breast, colon, and skin carcinoma *in vitro* and *in vivo* (5–7). $1,25(OH)_2D_3$ was also shown to induce differentiation of leukemic cells *in vitro* (8) and to prolong the survival time of leukemic animals *in vivo* (9). Despite its potent antitumor effect in a variety of human malignancies, the hypercalcemic side effect of $1,25(OH)_2D_3$ has prevented its clinical application, and earlier studies to demonstrate the antitumor effect of $1,25(OH)_2D_3$ *in vivo* were performed using mice maintained on the low-calcium diet (6, 7).

Recently a number of vitamin D_3 analogues have been developed in an effort to separate the calcemic activity and the antiproliferative/differentiation-inducing activity (10). We identified 22-oxa-$1,25(OH)_2D_3$, or 22-oxa-calcitriol, as an analogue that is more potent than $1,25(OH)_2D_3$ in inhibiting proliferation of leukemic cells but is much weaker than $1,25(OH)_2D_3$ in calcemic activity (11, 12). The molecular structure of OCT differs from that of $1,25(OH)_2D_3$ only in that an oxygen atom is substituted for a carbon atom in the side chain skeleton. As the first step toward clinical application of OCT for the treatment of human malignancies, we tested the antiproliferative effect of OCT in ER-positive and ER-negative breast cancer cells *in vitro* and demonstrated that OCT was a potent inhibitor of breast cancer cell growth regardless of ER status (13). In the present *in vivo* study, we intended to examine the effect of systemic administration of OCT on the growth of human breast carcinoma implanted into athymic mice, with the specific aims of (*a*) determining whether OCT had the ability to suppress the growth of ER-negative as well as ER-positive tumors *in vivo* without causing hypercalcemia and (*b*) determining whether the antitumor effect of OCT could be enhanced by combined therapy with other drugs, especially the antiestrogen tamoxifen in the case of ER-positive tumors.

MATERIALS AND METHODS

Materials. Tamoxifen and Adriamycin were purchased from Sigma Chemical Co. (St. Louis, MO), and estradiol dipropionate was from Teikoku-Zouki Co. (Tokyo, Japan). OCT was synthesized in our laboratory as described previously (14).

Animals and Tumors. Female athymic mice at 5 weeks of age were purchased from Clea Japan, Inc. (Tokyo, Japan). An estrogen-dependent human breast carcinoma, MCF-7 (15), and an estrogen-independent tumor, MX-1 (16), were studied in the following experiments. A 2-mm^3 fragment of aseptically resected tumor was implanted s.c. into the subaxillary region of female athymic mice at 6 weeks of age, and animals were maintained in sterilized cages. Food and water were autoclaved and provided *ad libitum*. For MCF-7 tumor, 100 μg estradiol dipropionate was given s.c. on the day of tumor transplantation to promote tumor growth, and 3 days later treatment with administration p.o. of OCT or tamoxifen (dissolved in 100 μl propylene glycol) 5 times a week for 4 weeks was started. In some experiments the combined effect of OCT and tamoxifen was also examined. For MX-1 tumor, 3 days after tumor transplantation treatment with administration p.o. of OCT (dissolved in 100 μl propylene glycol) 3 times a week for 4 weeks was started. In this tumor the effect of OCT was compared with that of Adriamycin, which was dissolved

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[4] The abbreviations used are: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; OCT, 22-oxa-$1,25(OH)_2D_3$ (22-oxa-calcitriol); ER, estrogen receptor; BW, body weight.

in 100 μl of saline and injected i.p. At the end of each experiment, animals were sacrificed, and the tumor weight was measured after careful resection. Blood was drawn and pooled for each group to measure serum calcium concentrations by orthocresolphthalein complex method (13).

Cell Lines. Two well-characterized ER-positive human breast carcinoma lines, MCF-7 and ZR-75-1, were studied. MCF-7 cells were kindly provided by Dr. T. Kuroki (Institute of Medical Science, University of Tokyo, Tokyo, Japan), and ZR-75-1 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured and maintained in RPMI-1640 supplemented with 10% fetal calf serum (Gibco, Grand Island, NY). For experiments, cells were washed with phenol red-free and FCS-free modified Eagle's medium (Gibco) and plated at a density of 4×10^4 cells/35-mm culture dish in 3 ml of phenol red-free modified Eagle's medium supplemented with 10% charcoal-treated fetal calf serum. After cells were treated with various doses of OCT or tamoxifen, either singly or in combination, every other day for 8 days, cell number was determined using a Coulter counter (model ZM). OCT and tamoxifen were dissolved in 100% ethanol, and the final concentration of ethanol in the medium did not exceed 0.1%.

RNA Extraction and Northern Blot Analysis. Tumors were homogenized, and total cellular RNA was prepared by denaturation in guanidinium thiocyanate followed by pelleting through cesium chloride cushion (17). Northern blot analysis was performed using approximately 30 μg of polyadenylated RNA and a 2.1-kilobase *Eco*RI cDNA fragment for human vitamin D receptor (courtesy of Dr. J. Wesley Pike) (18).

Statistical Analysis. Data were expressed as the mean ± SEM, and the statistical significance of the differences was determined by one-way analysis of variance.

RESULTS AND DISCUSSION

Antitumor Effect of OCT in ER-positive MCF-7 Tumor. Antiestrogens such as tamoxifen have been widely used for endocrine therapy of breast cancer, especially ER-positive tumors (19). Thus we examined the antitumor effect of OCT in comparison with that of tamoxifen, using female athymic mice implanted with the well-characterized ER-positive human breast carcinoma MCF-7 (15), the growth of which was dependent on exogenously added 17β-estradiol. OCT or tamoxifen was given p.o. five times a week for 4 weeks, and the antitumor effect was evaluated based on their effect on tumor weight on day 28. As shown in Fig. 1*A*, OCT as well as tamoxifen suppressed the growth of MCF-7 tumor in a dose-dependent manner, and the antitumor effect of 1.0 μg/kg BW OCT (mean ± SEM of tumor weight in six animals: 28 ± 4% of vehicle-treated group) was comparable to that of 2.0 mg/kg BW tamoxifen (25 ± 6% of control group). The doses of OCT used in these experiments did not raise serum calcium levels in the treated animals (data not shown). These results indicate that OCT is as potent as tamoxifen in terms of its antitumor effect on ER-positive human breast carcinoma *in vivo*. Judging from the time course of their effects on tumor size, calculated from the length and the width of each tumor, it appeared that OCT and tamoxifen did not induce tumor regression but delayed the growth of MCF-7 tumor (data not shown).

Synergistic Antitumor Effect of OCT and Tamoxifen. We next examined whether the combined treatment with OCT and tamoxifen could enhance the antitumor effect by single-agent treatment. This possibility was first tested *in vitro* using two well-characterized ER-positive human breast cancer cell lines, MCF-7 and ZR-75-1. As shown in Fig. 2, OCT as well as tamoxifen inhibited the proliferation of the ER-positive breast cancer cells in a dose-dependent manner between 10^{-10} and 10^{-8} M. Neither OCT nor tamoxifen was cytotoxic, and their effects were primarily to slow the replication rate (data not shown).

When ZR-75-1 cells were treated with both 10^{-10} M tamoxifen, which had no antiproliferative effect, and 10^{-10} M OCT, which inhibited cell proliferation by only 15%, a decrease in cell number of more than 40% was observed (Fig. 2*A*). A synergistic antiproliferative effect of OCT and tamoxifen was also observed in MCF-7 cells (Fig. 2*B*). Based on these results of *in vitro* studies, we next carried out *in vivo* experiments. As shown in Fig. 1*B*, when animals bearing MCF-7 tumors were treated with both 0.01 mg/kg BW tamoxifen, which alone had virtually no antitumor effect, and 0.001 μg/kg BW OCT, which by itself suppressed tumor progression by 28%, a decrease in tumor weight of nearly 60% was observed.

It is well established that steroid hormones such as estrogen and $1,25(OH)_2D_3$ exert their biological functions through specific interaction with their cognate intracellular receptors, which in turn bind to the regulatory regions of their target genes and thus modulate the transcription rate (3). The DNA sequences called hormone-responsive elements have been defined for each steroid hormone at the molecular level (20). In addition, the mechanisms by which different steroid hormone receptors and transcription factors interact on the DNA sequences are being disclosed (21–23). Although the target genes responsible for the antiproliferative activity of $1,25(OH)_2D_3$/OCT and antiestrogens are not known, the current results that OCT and tamoxifen act synergistically to suppress the growth of breast carcinoma *in vitro* and *in vivo* raise the possibility that there is some cooperative interaction between their signaling pathways after binding to the receptors and/or between their interactions with the DNA sequences. In addition, from a clinical point of view, our results may have important implications since it is expected that a potent anticancer effect can be obtained by the combination of different modes of endocrine therapy.

Antitumor Effect of OCT in ER-negative MX-1 Tumor. ER status is known to be an important predictive factor for endocrine therapy of breast carcinoma. Patients with ER-negative tumors, who constitute approximately 30–40% of total patients, do not benefit from antiestrogen therapy and in fact have a poorer prognosis than those with ER-positive tumors (19). Systemic therapy for these ER-negative breast carcinomas has depended on intensive chemotherapy with adverse side effects. These cytotoxic drugs are obviously not suitable for patients suffering from concomitant myelosuppressive disorders. Ac-

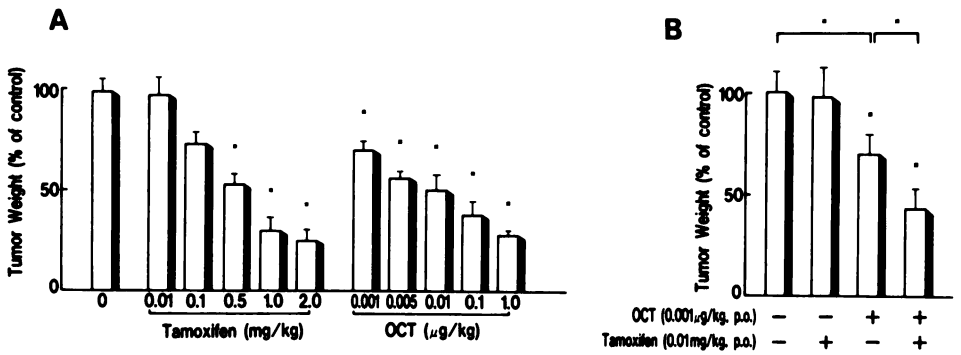

Fig. 1. Effect of administration p.o. of OCT and tamoxifen on the growth of ER-positive human breast carcinoma MCF-7 in athymic mice. The indicated doses of tamoxifen or OCT, either singly (*A*) or in combination (*B*), was given p.o. five times a week for 4 weeks to athymic mice implanted with ER-positive human breast carcinoma MCF-7. Tumor weight was measured on day 28, and data are expressed as a percentage of the control group and shown as the mean ± SEM ($n = 6$). *, significant difference from the control (vehicle-treated) group unless indicated otherwise. Similar results were obtained in three (*A*) and two (*B*) separate experiments.

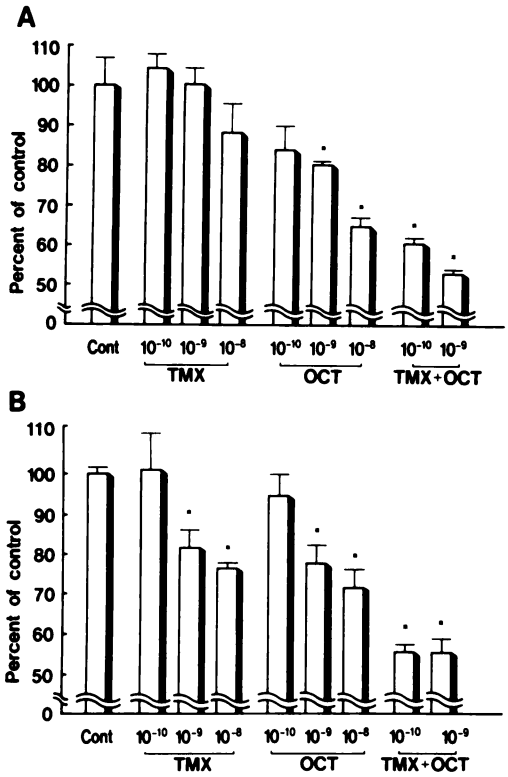

Fig. 2. Synergistic antiproliferative effect of OCT and tamoxifen in ER-positive breast cancer cells *in vitro*. ER-positive human breast carcinoma cells ZR-75-1 (*A*) and MCF-7 (*B*), were treated with the indicated doses of OCT and tamoxifen (*TMX*), either alone or in combination, every other day for 8 days, and the cell number was determined with a Coulter counter. Data are expressed as a percentage of the control (vehicle-treated) group and are shown as the mean ± SEM (n = 4). *, significant difference from the control group. The combined effect of OCT and TMX at either 10^{-10} or 10^{-9} M was significantly greater than the effect by a single agent of the respective dose. Similar results were obtained in three separate experiments.

tive vitamin D_3 compounds have no myelosuppressive effect and have been shown to induce differentiation of hematopoietic cells (8).

We have demonstrated in the previous *in vitro* studies (13) that OCT as well as $1,25(OH)_2D_3$ inhibits the proliferation of breast cancer cells *in vitro* regardless of ER status. To extend these observations *in vivo*, the antitumor effect of systemic administration of OCT was examined in athymic mice implanted with ER-negative human breast carcinoma MX-1, which grew rapidly without exogenous estrogen. OCT was given p.o. three times a week, and the antitumor effect was assessed on day 28 according to the direct measurement of tumor weight. As shown in Fig. 3*A*, OCT suppressed the growth of MX-1 tumor in a dose-related fashion between 0.01 and 1.0 μg/kg BW, but the effect of OCT seemed not to induce tumor regression but to slow the growth of MX-1 tumor (data not shown). When the antitumor effect of OCT was compared with that of Adriamycin, one of the most potent cytotoxic drugs as a single agent for the treatment of human malignancies including breast cancer, it appeared that 1.0 μg/kg BW OCT was more potent than 500 μg/kg BW Adriamycin administered i.p. (Fig. 3*B*). Again, OCT did not raise serum calcium levels in the treated animals (data not shown). These results suggest that OCT may also be a powerful tool for the systemic adjuvant therapy of ER-negative breast carcinoma for which conventional antiestrogen treatment is not effective.

In order to determine whether VDR was present in the tumors used in the present study, the expression of VDR mRNA was examined by Northern blot analysis. A single transcript corresponding to the reported 4.4-kilobase VDR mRNA in HL-60 cells (18) was observed in both MX-1 and MCF-7 tumors (data not shown). Based on these results, it is plausible to assume that the antitumor effect of OCT is mediated, like the parent compound, through interaction with VDR in the tumors. The reported observations that the antitumor effect of $1,25(OH)_2D_3$ correlated well with the presence and the level of VDR in tumors also support this concept (6). With respect to the mechanism by which $1,25(OH)_2D_3$ and OCT suppress tumor growth *in vivo*, it is intriguing to note that OCT is a potent inhibitor of angiogenesis (24), which plays an important role in tumor growth. However, further studies are required to prove the presence of functional VDR protein in the tumors and to determine whether the antitumor effect of OCT as well as that of the parent compound is mediated through specific interaction with VDR in the tumors. It is possible that the vitamin D compounds affect tumor growth indirectly via their effects on host-derived factors, especially those in the immune system.

It also remains to be clarified why the substitution of an oxygen atom for a carbon atom converts $1,25(OH)_2D_3$ to an analogue that has virtually no calcemic activity and yet retains potent antitumor activity. It has been demonstrated that OCT has much less affinity than $1,25-(OH)_2D_3$ for plasma vitamin D binding protein *in vivo* as well as *in vitro* and is cleared very quickly from the circulation into the bile (25, 26). Thus preferential distribution and uptake of OCT into certain tissues not involved in calcium metabolism may partly account for its selective biological activities *in vivo*. The observation that OCT is almost equipotent to $1,25(OH)_2D_3$ in terms of activating osteocalcin gene transcription (27) further supports pharmacokinetic differences *in vivo* rather than qualitative differences in their genomic actions. Future studies to clarify the detailed mechanism of action and metabolism of OCT will make it possible to deliver the compound efficiently to the target tissues and further enhance its anticancer effects.

In summary, we have demonstrated in the present study using athymic mice implanted with human breast carcinoma that OCT has a potent antitumor effect not only in ER-positive but also ER-negative human breast carcinoma *in vivo*. The potency of OCT is comparable to that of tamoxifen in an ER-positive tumor or Adriamycin in an ER-negative tumor. Furthermore, in an ER-positive MCF-7 tumor a synergistic antitumor effect of OCT and tamoxifen is observed. Taken together with the high frequency of VDR in breast carcinoma, these results suggest that OCT may provide a new strategy, either alone or in combination with other anticancer drugs, for the treatment of breast carcinoma regardless of ER status. The presence of VDR in a wide variety of malignant cells suggests that OCT may be a valuable agent for endocrine therapy of other human malignancies as well (28).

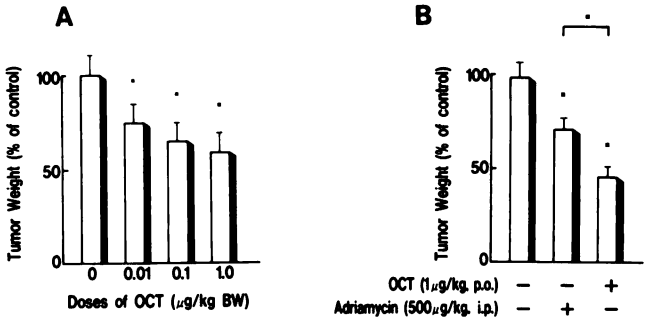

Fig. 3. Effect of administration p.o. of OCT and Adriamycin on the growth of ER-negative human breast carcinoma MX-1 in athymic mice. In *A*, the indicated dose of OCT was given p.o. three times a week for 4 weeks to athymic mice implanted with ER-negative human breast carcinoma MX-1. Animals were sacrificed on day 28, and the tumor weight was measured. Data are expressed as a percentage of the control (vehicle-treated) group and shown as the mean ± SEM (n = 10). *, significant difference from the control group. Similar results were obtained in three separate experiments. In *B*, OCT (1.0 μg/kg BW) or Adriamycin (500 μg/kg BW) was given p.o. or i.p., respectively, three times a week for 4 weeks, and tumor weight was measured on day 28. Data are expressed as a percentage of the control (vehicle-treated) group and shown as the mean ± SEM (n = 10). *, significant difference from the control group unless indicated otherwise. Similar results were obtained in two separate experiments.

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