Expression of Cyclin D1, Cyclin E, p21^Cip1 and p27^Kip1 in Chemically Induced Rat Mammary Tumor Treated with Tamoxifen and Transforming Growth Factor-β1

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Background: Tamoxifen (TAM) inhibits the action of estrogen by binding to estrogen receptors, and also has non-estrogen receptor mediated cytostatic activities. Transforming growth factor-β1 (TGF-β1) inhibits the proliferation of many other cell types, such as epithelial, hematopoietic and endothelial cells. Methods: We investigated the effects of tamoxifen on the growth of 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat mammary tumors and the expression of cyclin D1, cyclin E, p21^Cip1, and p27^Kip1 by performing immunohistochemistry and Western blot analysis, and studied whether TGF-β1 injection amplified the effects of TAM. When tumor size reached between 10-15 mm in the largest dimension, the rats were divided into 3 groups: DMBA-control group (n=12), DMBA-TAM group (n=14) and DMBA-TAM plus TGF-β1 group (n=5). Results: The consecutive administration of TAM markedly decreased the tumor development compared with the DMBA-control group. The DMBA-TAM and DMBA-TAM plus TGF-β1 groups showed decreased expression of bromodexoyuridine, cyclin D1, cyclin E, and p21^Cip1 when compared with those of the DMBA-control group. On the other hand, the labeling index of p27^Kip1 was higher in the DMBA-TAM plus TGF-β1 group than in the DMBA-control group. Conclusions: TAM suppresses tumor development, which may be associated with down-expression of cyclin D1 and cyclin E, and overexpression of p27^Kip1, and addition of TGF-β1 does not influence tumor development treated by TAM.

Tamoxifen (TAM) was first evaluated for the palliative treatment of advanced breast cancers.¹ Today, long-term TAM therapy remains as an endocrine treatment for selected patients with all stages of breast cancer.² TAM inhibits the action of estrogen by binding to estrogen receptors (ER),³ which is associated with the inhibition of mitosis by arresting the cells in the G0 and G1 phases,⁴ but it is now apparent that TAM also performs non-ER mediated cytostatic activities.⁵ This involves the inhibition of protein kinase C and calmodulin-dependent cAMP phosphodiesterase as well as the regulation of peptides such as insulin-like growth factor 1 and transforming growth factor-β (TGF-β).⁶ TGF-β secretory enhanced by anti-estrogen treatment was previously shown in vitro in ER-positive MCF-7 cells,⁷ and in ER-negative human fetal fibroblasts.⁸ In addition, TAM treatment in vivo also increased the level of extracellular TGF-β1 in human primary breast cancers.⁹ TGF-β1 inhibits the proliferation of many other cell types, such as epithelial, hematopoietic and endothelial cells.⁹ TGF-β1 appears to be an important negative regulator of breast cancer cell growth.⁹ TGF-β1-induced inhibitory signal involves down-regulation of transcription, decreased phosphorylation of target proteins, and inactivation of cell cycle-regulated enzymes.⁹

Cell cycle progression in mammalian cells are regulated by the interactions of cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors (CDKI). Cyclin D1 binds to and activates its major catalytic partners, CDK4 and
CDK6, which can in turn phosphorylate the Rb tumor suppressor proteins.\textsuperscript{10} Cyclin E binds to and activates CDK2, which also phosphorylates the Rb proteins.\textsuperscript{10} CDKIs generally inhibit the cell cycle progression by regulating the cyclin-CDK complexes. The Cip/Kip family (such as p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1} and p57\textsuperscript{Kip2}) inhibit the kinase activities of pre-activated G1 cyclin E-CDK2, cyclin D-CDK4/6 and other cyclins.\textsuperscript{10}

It is known that 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat mammary tumors are generally strongly hormone-dependent for both induction and growth.\textsuperscript{11} Moreover, overexpression of cyclin D1 and cyclin E has been found in carcinogen-induced rat mammary tumors,\textsuperscript{12} and recently, we have observed increased expression of cyclin D1, cyclin E, and p21\textsuperscript{Cip1} associated with decreased expression of p27\textsuperscript{Kip1} in DMBA-induced rat mammary carcinogenesis.\textsuperscript{13} On the other hand, TAM has reduced mammary tumor induction and growth in animal models.\textsuperscript{11} However, supportive in vivo studies about the effects of TAM treatments on the alteration of G1 cell cycle regulatory proteins are lacking. In this study, we investigated the effects of TAM on the growth of DMBA-induced rat mammary tumors and the expression of cyclin D1, cyclin E, p21\textsuperscript{Cip1}, and p27\textsuperscript{Kip1}; and studied whether TGF-β1 injection amplified the effects of TAM.

**MATERIALS AND METHODS**

**Animals and treatment**

Forty-one-day-old female Sprague-Dawley rats (Kist, Taejun, Korea) were used. Throughout the experiment, all of the rats were housed under a controlled environment with 12 hr light/dark cycles and temperature of 22 ± 2°C, given a commercial diet with tap water ad libitum, and weighed every 7 days. After a 1 week acclimatization period, the rats were divided into two groups: a group of 50 tumor-induced rats received an intragastric dose of 10 mg of DMBA (Sigma Chemical Co., St Louis, MO, U.S.A.) in 1.0 mL of normal saline into the tail vein 1 hour before autopsy. Responses were graded as complete (if the tumor disappeared), partial (if there was a greater than 50% reduction of tumor volume), and stable (if there was a less than 50% reduction or an increase of less than 25% of volume). Progressive disease was defined as the appearance of any new lesions or an increase of greater than 25% of tumor volume.

All mammary tumors were dissected and fixed as follows: half of the tumor was fixed in 10% neutral buffered formalin for histological study and immunohistochemistry, and the other half of the tumor was immediately frozen in liquid nitrogen and stored at -70°C for Western blot analysis.

**Histology and immunohistochemistry**

For histological evaluation, the formalin-fixed tissues were embedded in paraffin, cut at 4 μm and stained with hematoxylin and eosin. Serial sections of 4 μm thickness were made and spread on poly-L-lysine coated slides. Paraffin sections were immersed in three changes of xylene and hydrated using a graded series of alcohols. Antigen retrieval was routinely performed by immersing the sections in a 0.01 M citrate buffer (pH 6.0) in a pressure cooker by autoclaving for 15 min. Sections were blocked with normal horse serum at 37°C for 30 min and then incubated with a primary antibody for 1 hour at room temperature. Primary antibodies that were used were as follows: monoclonal mouse anti-BrdU (DAKO, Santa Babara, CA, U.S.A.) at

When the tumor size reached between 10-15 mm in the largest dimension, the rats were divided into 3 groups (day 1): a DMBA-control group (n=12), a DMBA-TAM group (n=14) and a DMBA-TAM plus TGF-β1 group (n=5). TAM (Sigma Chemical Co., St Louis, MO, U.S.A.) treatment was started on day 1 and continued for the next 30 days. Eight mg/kg body weight of TAM in 0.1 mL sesame oil was injected subcutaneously into the animals’ backs. Recombinant human TGF-β1 (R&D Systems Inc., Minneapolis, MN, U.S.A.) was dissolved in 4 mM HCl containing 1 mg of bovine serum albumin per milliliter. Two μg/mL of TGF-β1 was injected into the tail vein at day 28 of TAM treatment, and this continued for 3 days. The DMBA-control group was subcutaneously injected with the same volume of sesame oil alone. The rats were sacrificed on day 31. Rats were given a single intravenous injection of 0.1 mg/g body weight of BrdU (Sigma Chemical Co., St Louis, MO, U.S.A.) in 1.0 mL of normal saline into the tail vein 1 hour before autopsy. Responses were graded as complete (if the tumor disappeared), partial (if there was a greater than 50% reduction of tumor volume), and stable (if there was a less than 50% reduction or an increase of less than 25% of volume). Progressive disease was defined as the appearance of any new lesions or an increase of greater than 25% of tumor volume.
1:100 dilution, rabbit polyclonal anti-human cyclin D1 (Upstate Biotechnology, Inc., Lake Placid, NY, U.S.A.) at 1:200 dilution, rabbit polyclonal anti-rat cyclin E (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at 1:100 dilution, rabbit polyclonal anti-human p21^Cip1 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at 1:200 dilution, and rabbit polyclonal anti-human p27^Kip1 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at 1:200 dilution. Staining was achieved with a LSAB+ kit (DAKO, Santa Barbara, CA, U.S.A.) and developed with 3, 3' -diaminobenzidine tetrahydrochloride (Zymed Laboratories, Inc., San Francisco, CA, U.S.A.). Sections were counterstained for 5 min with Mayer’s hematoxylin and then mounted. Human breast cancer with intense staining for either cyclin D1, cyclin E, p21^Cip1, or p27^Kip1 was used as positive controls. Primary antibodies were omitted as negative controls.

Quantitation of Immunohistochemistry

Cells were considered positive for BrdU, cyclin D1, cyclin E, p21^Cip1, and p27^Kip1 when clear staining in the nucleus could be identified. Ten fields were randomly selected and the positive epithelial cells were counted. Labeling indices for BrdU, cyclin D1, cyclin E, p21^Cip1 and p27^Kip1 were calculated as percentage values, taking the total number of examined cells into account. At least 1,000 epithelial cells were counted throughout the entire area of tumor.

Western blot analysis

Frozen tissue from each tumor was sonicated according to Sgambato et al. The supernatants were assayed for protein content according to the Bradford method and were stored at -70°C until used. Fifty micrograms of protein extracts were run in a NuPAGE 4-12% Bis-Tris polyacrylamide gel (NOVEX, San Diego, CA, U.S.A.) and electro-blotted onto a nitrocellulose membrane (BioRad, Richmond, CA, U.S.A.) according to the manufacturer’s instructions. The blots were then blocked overnight at 4°C and incubated for 2 hours at room temperature with cyclin D1, cyclin E, and p21^Cip1 at 1:500 dilution, respectively. After washing with TBS-0.05% Tween 20, the blots were incubated for 45 min at room temperature with a secondary antibody (DAKO) at 1:1000 dilution, then after washing, they were developed with Western blotting chemiluminescence luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for X-ray examination.

Statistical analysis

Statistical association was performed using the SPSS 6.0 program. Data were shown as mean ±SD. The data for the grading of the treatment responses were statistically evaluated by using the Chi-square test. The change of tumor number and the labeling indices of BrdU, cyclin D1, cyclin E, p21^Cip1, and p27^Kip1 were compared among groups by using the one-way analysis of variance.

RESULTS

Treatment response and histology of mammary tumors

Tumors occurred only in the DMBA-treated group. At the beginning of TAM treatment, the number of tumors per rat were 1.50 ± 0.80 in the DMBA-control group, 2.36 ± 1.45 in the DMBA-TAM group, and 5.20 ± 3.42 in the DMBA-TAM plus TGF-β1 group. At the end of treatment, the number of tumors per rat was 4.33 ± 1.67 in the DMBA-control group, 2.00 ± 1.41 in the DMBA-TAM, and 4.40 ± 3.21 in the DMBA-TAM plus TGF-β1 group. Response rate was 1.9% in the DMBA-control group, 48.6% in the DMBA-TAM group, and 40.7% in the DMBA-TAM plus TGF-β1 group (Table 1). Thus, the consecutive administration of TAM markedly decreased the tumor development compared with the DMBA-control group (p < 0.05), and addition of TGF-β1 did not influence tumor development treated by TAM.

Tumors were classified histologically as 52 carcinomas in the DMBA-control group, 24 carcinomas and 4 benign tumors in the DMBA-TAM group, and 18 carcinomas and
4 benign tumors in the DMBA-TAM plus TGF-$\beta$1 group. The majority of tumors in the DMBA-control group revealed solid and papillary patterns and were composed of hyperplastic epithelial cells with varying degrees of cytologic atypia. The growth patterns of tumors in the DMBA-TAM and DMBA-TAM plus TGF-$\beta$1 groups was similar to that of untreated tumors, and well differentiated glands were observed frequently in the treated groups.

**Immunohistochemical staining of BrdU, cyclin D1, cyclin E, $p21^{\text{Cip1}}$, and $p27^{\text{Kip1}}$**

The topological staining patterns of BrdU, cyclin D1, cyclin E, $p21^{\text{Cip1}}$, and $p27^{\text{Kip1}}$ showed correlations among each other, as assessed by staining consecutive tissue sections (data not shown). On the other hand, the staining patterns of $p27^{\text{Kip1}}$ were not always concordant with that of the others; that is, $p27^{\text{Kip1}}$ was expressed concurrently with BrdU, cyclin D1, cyclin E, and $p21^{\text{Cip1}}$ in some areas, as well as independently in other areas (data not shown). The DMBA-TAM and DMBA-TAM plus TGF-$\beta$1 groups showed the decreased labeling indices of BrdU, cyclin D1, and cyclin E when compared with the DMBA-control group ($p<0.05$) (Table 2) (Fig. 1). The labeling index of $p21^{\text{Cip1}}$ slightly decreased in the treated group, however it is not statistically significant ($p<0.05$) (Table 2). On the other hand, the labeling index of $p27^{\text{Kip1}}$ was higher in the DMBA-TAM plus TGF-$\beta$1 than in the DMBA-control group ($p<0.05$) (Table 2).
2) (Fig. 2). The Immunohistochemical parameters mentioned above did not reveal significant differences between the DMBA-TAM and the DMBA-TAM plus TGF-1 groups.

Western blot analysis of cyclin D1, cyclin E and p21Cip1

As shown in Fig. 3, the DMBA-TAM group revealed markedly decreased expression of cyclin D1 and p21Cip1 when compared with the DMBA-control group. Interestingly, cyclin D1 expression was increased in the DMBA-TAM plus TGF-1 group compared with the DMBA-TAM group. Cyclin E expression was slightly decreased in the treated group when compared with the DMBA-control group. Several isoforms of cyclin E, which ranged between 28 kD and 51 kD, were detected.

DISCUSSION

In this study, the growth of TAM-treated tumors was repressed when compared with that of the DMBA-control group. This is in accordance with previous study.14 TAM induced accumulation of MCF-7 human mammary carcinoma cells in the G0/G1 phases of the cell cycle, and the percentage of BrdU-immunoreactive cells were more decreased in TAM-treated rat mammary tumors than in untreated control tumors.14 Therefore, the repression of tumor development in the TAM-treated rat in this study may be caused by decreased cell proliferation as proved by the reduction of BrdU-labeling index in the TAM-treated rat.

Estrogen stimulated the resting cells to enter the cell cycle and accelerated the G1-S phase progression through the increased expression of cyclin D1 and c-Myc.25 In addition, steroidal anti-estrogen ICI 182780 in breast cancer cells resulted in the reduction of cyclin D1 mRNA and protein,16 and estrogen withdrawal in MCF-7 human breast cancer xenografts showed the reduction of cyclin D1.17 These findings support the reduction of cyclin D1 in the TAM-treated tumors. Interestingly, the DMBA-TAM plus TGF-1 group showed increased expression of cyclin D1 when compared with the DMBA-TAM group. TGF-β1 treatment of human colon carcinoma cells resulted in the rapid down-regulation of c-myc RNA and protein level.18 In addition, expression of c-Myc protein repressed the expression of cyclin D1 at the level of transcription initiation in BALB/c-3T3 mouse fibroblasts,19 and TGF-β1 treatment increased cyclin D1 mRNA and protein level in COLO-357 human pancreatic cancer cells.20 These effects of TGF-β1 on cyclin D1 may explain our results. On the contrary, TGF-β1 inhibited the expression of cyclin D1 in rat intestinal epithelial cells21 and human melanoma cells.22 At present, however, no satisfactory explanation for this observation can be given.

Several isoforms of cyclin E were detected in this study, as previously reported in human breast cancers23 and mouse mammary tumor development.24 The cause of these alterations was suggested to be due to the gene amplification or the alternative splicing of cyclin E mRNA.25 We quantitated the optical density of 51 kD isoform of cyclin E because the other isoforms were reported to be lacking in any CDK2-associated kinase activities.24 The anti-estrogenic effect of TAM cannot explain the results showing the decreased expression of cyclin E in the TAM-treated tumors, because estrogen activates the cyclin E-CDK2 complex without alteration in the expression level.26 In addition, TAM did not alter the expression level of cyclin E in MCF-7 cells.27 TAM induced the autocrine secretion of TGF-β1 in human breast cancer cells,56 as well as human fetal fibroblasts.7 Moreover, TAM treatment in vivo increased the level of extracellular TGF-β1 in human primary breast can-
cancers. TGF-β1 treatment in human keratinocytes induced down-regulation of cyclin E mRNA and protein. Therefore, this action of TGF-β1 may explain our results which show decreased expression of cyclin E in the TAM-treated tumor.

The p21Cip1 expression level in Western blot analysis was decreased when compared with the untreated group, without a significant difference between the DMBA-TAM and the DMBA-TAM plus TGF-β1 groups. This finding suggests that TGF-β1 is not involved in the decreased expression of p21Cip1. However, the up-regulation of p21Cip1 by TGF-β1 has been demonstrated in malignant melanoma cells. Moreover, TAM did not alter the p21Cip1 expression level in MCF-7 human breast cancer cells. This discrepancy could be explained in two ways: first, the expression of p21Cip1 is closely associated with the expression of cyclin D1, and second, a feedback inhibitory loop exists to maintain homeostatic control between positive- and negative-acting factors involved in the cell cycle progression. At present, however, no satisfactory explanation for this observation can be given.

The p27Kip1 expression level was increased in the TGF-β1-arrested WM35 human melanoma cells. Moreover, anti-estrogen increased the expression of p27Kip1 in MCF-7 human breast cancer cells and estrogen withdrawal in MCF-7 human breast cancer xenografts showed the increased expression of p27Kip1. It thus appears that the increased expression of p27Kip1 in the DMBA-TAM plus TGF-β1 group is caused by the combined effects of TAM and TGF-β1. However, we cannot exclude the possibility that this observation may be caused by the result of cytostasis induced by TAM and TGF-β1 because the level of p27Kip1 ubiquitination is decreased in quiescent cells compared with proliferating cells. Well differentiated glands expressing only p27Kip1 were frequently observed in the treated tumors. This result can be explained by the fact that p27Kip1 protein levels have been shown to increase under the conditions of differentiation. Some areas showed concurrent expression of BrdU, cyclin D1, cyclin E, p21Cip1, and p27Kip1. Weinstein et al. postulated the existence of a feedback inhibitory loop between cyclin D or cyclin E and p27Kip1 based on the observation that overexpression of cyclin D1 and cyclin E have been associated with the expression level of p27Kip1, and hypothesized that its function was to maintain homeostatic control between positive- and negative-acting factors involved in the cell cycle progression, in order to prevent the potential toxic effects of excessive cyclin D1/CDK or cyclin E/CDK kinase activity.

There are two defects in the technical aspect of our results. First, we studied the response rate and expression of G1 cell cycle regulatory proteins at the dose of 8 mg/kg body weight of TAM and 2 μg/mL of TGF-β1, and cannot exclude the possibility that different results may occur when different dosages of TAM or TGF-β1 is injected into animals. Second, the examination of TGF-β1 expression can explain the effect of TGF-β1 on tumors of experimental groups, however, we did not investigate TGF-β1 expression. Therefore, further studies based on the dosage and expression of TGF-β1 will be necessary in order to evaluate the effect of TGF-β1 on tumor behavior.

In conclusion, TAM suppresses tumor development, which may be associated with down-expression of cyclin D1 and cyclin E, and overexpression of p27Kip1, and addition of TGF-β1 does not influence tumor development treated with TAM. These observations suggest the feasibility of therapeutic concepts aimed at decreasing cell proliferation via the arrest of the cell cycle by administration of a regulator of the cell cycle.

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