Novel Tumor-promoting Property of Tamoxifen

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Abstract
The tumor-promoting phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate) cooperates with c-Src overexpression to transform rat fibroblasts. TPA transforms c-Src-overexpressing cells by depleting the δ isoform of protein kinase C (PKCδ). Tamoxifen, which has both estrogen-mimetic and estrogen-antagonist properties, has been widely used to improve the prognosis of breast cancer patients. However, with extended use, there is an increased risk for endometrial and other cancers that can be observed within 10 years of treatment. We report here that tamoxifen, similar to TPA, cooperates with c-Src overexpression to transform 3Y1 rat fibroblasts. Tamoxifen induced both DNA synthesis and anchorage-independent cell proliferation in c-Src-overexpressing, but not in parental, 3Y1 rat fibroblasts. Tamoxifen also induced an association between c-Src and PKCδ that resulted in the tyrosine phosphorylation and down-regulation of PKCδ. These phenotypes were not induced by estrogen, indicating that the effect of tamoxifen was in addition to any estrogen-mimetic effects. Thus, in addition to the hyperplasia-inducing capability of an estrogen-mimetic, tamoxifen has an additional tumor-promoting capability similar to that of TPA. The dual tumor-promoting capability of both estrogen- and TPA-mimetic properties for tamoxifen may contribute to the increased incidence of endometrial cancers observed in the relatively short exposure period of <10 years.

Introduction
Tamoxifen, a nonsteroidal mixed estrogen agonist/antagonist, is widely used as first-line therapy for patients suffering from metastatic as well as primary breast carcinoma (1). Tamoxifen has also been shown to reduce the incidence of primary breast carcinoma in women who are at high risk for developing this disease (2), and prophylactic administration of tamoxifen has been suggested for these high-risk patients (3). Five-year tamoxifen treatment substantially improves prognosis for breast cancer patients; however, 10-year treatment showed no significant improvement and substantially increased the risk for endometrial cancer and possibly gastrointestinal cancers as well (4–7). The increase in endometrial cancer has been widely attributed to an estrogen mimetic effect of tamoxifen in endometrial tissues where both estrogen and tamoxifen induce hyperplasia (8). However, an increase in cancer incidence within 10 years of exposure to tamoxifen suggests that tamoxifen possesses a tumor-promoting capability beyond the estrogen-mimetic properties of tamoxifen.

We demonstrated previously that the tumor-promoting phorbol ester TPA4 cooperates with c-Src to transform 3Y1 rat fibroblasts (9). In this cell culture model system, 3Y1 rat fibroblasts overexpressing c-Src (3Y1c-Src cells) became transformed when treated with TPA. The effect of TPA could be mimicked by inhibitors of the δ isoform of PKC (PKCδ), indicating that the effects of TPA were attributable to its ability to down-regulate PKCδ (9, 10). Consistent with these results, transgenic mice overexpressing PKCδ in their epidermis were reported recently to be resistant to skin tumor promotion by TPA (11). Thus, the 3Y1c-Src cells appear to accurately reflect the tumor-promoting effects of TPA, with c-Src being the initiating mutation and PKCδ down-regulation the promoting effect in a two-stage tumorigenesis model. The 3Y1c-Src cells are therefore capable of detecting tumor-promoting compounds able to cooperate with a signaling oncogene, such as an overexpressed tyrosine kinase, a common genetic defect in human cancers (12), to transform cells.

Because of the likelihood that tamoxifen has tumor-promoting properties beyond its estrogen-mimetic capability, we examined the effect of tamoxifen on the 3Y1c-Src cells. We report here that tamoxifen, similar to TPA, causes the down-regulation of PKCδ and has tumor-promoting effects that cooperate with an overexpressed tyrosine kinase to transform rat fibroblasts.

Results
Tamoxifen and TPA Induce DNA Synthesis in 3Y1c-Src but not Parental 3Y1 Cells. We first examined the ability of tamoxifen to stimulate DNA synthesis in both 3Y1c-Src and parental 3Y1 cells after serum withdrawal. Subconfluent cultures of 3Y1 or 3Y1c-Src cells maintained in medium contain-
ing 10% serum were transferred to medium containing 0.5% serum for 36 h. At this point, both the 3Y1 and 3Y1c-Src cells exhibited minimal uptake of [3H]thymidine. Tamoxifen was then added, and DNA synthesis was monitored by [3H]thymidine uptake. As shown in Fig. 1A, tamoxifen stimulated an increase in DNA synthesis in the 3Y1c-Src but not in the parental 3Y1 cells. The increase was observed between 12 and 20 h after treatment. For comparison, we also examined the effect of TPA on DNA synthesis in these cells. As shown in Fig. 1B, TPA stimulated a biphasic increase in DNA synthesis in the 3Y1c-Src cells with an increase in [3H]thymidine uptake between 2 and 4 h and another between 12 and 20 h. The biphasic increase in DNA synthesis suggested that serum deprivation of 3Y1c-Src cells results in two populations of cells that are arrested in different places in the cell cycle. We did not observe the short-term increase in DNA synthesis using tamoxifen, indicating that TPA may have effects beyond those of tamoxifen. The tamoxifen-induced increase in DNA synthesis was dose dependent and could be detected at concentrations as low as 0.1 μM tamoxifen, with maximal levels seen between 2 and 5 μM (Fig. 1C). Importantly, this level of tamoxifen is achieved in the serum of individuals on standard 20 mg/day protocols (13).

Estrogen was unable to stimulate DNA synthesis in either the 3Y1 or 3Y1c-Src cells (data not shown). As shown in Fig. 2, the 3Y1c-Src cells express detectable levels of the β estrogen receptor but not the α estrogen receptor. Thus, although the 3Y1c-Src cells do contain estrogen receptor β, the effects of tamoxifen go beyond estrogen mimetic effects.

**Tamoxifen Induces Anchorage-independent Growth in c-Src-overexpressing but not Parental 3Y1 Cells.** We next examined the effect of tamoxifen upon anchorage-independent growth as measured by the ability to form colonies in agar suspension. 3Y1 and 3Y1c-Src cells were seeded into agarose, and colony-forming efficiency was determined in the presence of either tamoxifen (5 μM; A) or TPA (400 nM; B) for the indicated times. The bars represent the SD for the fold effect of tamoxifen and TPA treatments as shown. All experiments were performed at least two times.

**Fig. 1.** Tamoxifen and TPA induce DNA synthesis in 3Y1c-Src but not parental 3Y1 cells. Subconfluent 3Y1 and 3Y1c-Src cells were placed in medium containing 0.5% serum for 36 h and then treated with either tamoxifen (5 μM; A) or TPA (400 nM; B) for the indicated times. Values were normalized to the cpm/cell for the untreated time zero controls, which were given a value of 1. Actual cpm/cell values were 8.8 ± 3.10³ for the 3Y1 cells and 4.0 ± 10⁻³ for the 3Y1c-Src cells. Relative [3H]thymidine incorporation values were obtained from three independent experiments. C, the dose response to tamoxifen for induction of DNA synthesis was examined by doing a 1-h [3H]thymidine pulse at 20 h after addition of tamoxifen at the concentrations shown. Bars, the SD for the fold effect of tamoxifen and TPA treatments as shown. All experiments were performed at least two times.

**Fig. 2.** Estrogen receptor levels in 3Y1c-Src cells. Lysates from 3Y1 (Lane 1) and 3Y1c-Src (Lane 2) cells were analyzed for estrogen receptor α and β levels as shown by Western blot analysis as described in “Materials and Methods.” As a positive control for estrogen receptor α, rat uterus tissue was used (Lane 3).

**Tamoxifen Induces Tyrosine Phosphorylation and Down-Regulation of PKCδ in Cells Overexpressing c-Src.** As described above, the tumor-promoting effect of TPA was the result of depleting cells of PKCδ (9). PKCδ has been implicated as a tumor suppressor gene in a variety of cell and animal systems (9–11, 14–17). Importantly, in cells transformed by v-Src, PKCδ was shown to associate with v-Src and become phosphorylated on tyrosine (18). The tyrosine phosphorylation of PKCδ by v-Src leads to reduced PKCδ levels (15), further indicating that reduced levels of PKCδ are efficiency of colony formation in the 3Y1c-Src cells (9). Tamoxifen similarly induced a 5-fold increase in colony-forming efficiency (Fig. 3A). Tamoxifen had no effect upon the colony-forming efficiency in the parental 3Y1 cells (Fig. 3A). The dose dependence for colony formation was similar to that observed for DNA synthesis (Fig. 3B). These data further support the hypothesis that tamoxifen has tumor-promoting properties similar to those of TPA.
critical for transformation. As shown in Fig. 4A, tamoxifen stimulated tyrosine phosphorylation of PKCδ in the 3Y1c-Src cells but not the parental 3Y1 cells. The level of tyrosine phosphorylation was comparable with that seen in v-Src-transformed cells (Fig. 4A). The effect was dose dependent with a dose response similar to that observed for DNA synthesis and colony formation with induction occurring at concentrations as low as 0.1 μM tamoxifen (Fig. 4B). As observed for DNA synthesis, estrogen did not stimulate tyrosine phosphorylation of PKCδ in the 3Y1c-Src cells (not shown). We next examined whether we could detect c-Src in PKCδ immunoprecipitates, and as shown in Fig. 4C, tamoxifen treatment caused PKCδ to coimmunoprecipitate with c-Src. These data indicate that tamoxifen stimulates association between overexpressed c-Src and PKCδ, leading to the phosphorylation of PKCδ on tyrosine. Because tyrosine phosphorylation of PKCδ by v-Src causes degradation of PKCδ (15), we examined the effect of tamoxifen upon the level of PKCδ. As shown in Fig. 4D, tamoxifen treatment reduced PKCδ levels to those seen in v-Src-transformed cells. Tamoxifen treatment did not reduce the level of PKCδ comparably in the v-Src-transformed cells (Fig. 4D), indicating that only a subpopulation of PKCδ is involved. Tamoxifen had little or no effect upon the level of PKCδ in the parental 3Y1 cells (Fig. 4D). We next examined the effect of tamoxifen upon PKCδ activity, and as shown in Fig. 4E, tamoxifen reduced the PKCδ activity (19) in the 3Y1c-Src cells but not the parental cells. The level of reduced PKCδ activity seen in Fig. 4E was quantitatively similar to the reduced level of PKCδ protein seen in Fig. 4D. Thus, in response to tamoxifen, as with TPA (9, 10), there are reduced levels of both PKCδ protein and activity. Although PKCδ levels are not reduced to the levels achieved by TPA treatment, this is likely because of an effect that was restricted to a subpopulation of PKCδ that associates with c-Src. However, this subpopulation is likely to be a critical subpopulation of PKCδ.

**Tamoxifen Induces PLD Activity in 3Y1 Cells Overexpressing c-Src.** We reported previously that inhibition or down-regulation of PKCδ led to an elevation of PLD activity in the 3Y1c-Src cells (9) and in 3Y1 cells overexpressing the epidermal growth factor receptor (10). Moreover, we went on to demonstrate that PLD1 overexpression could cooperate with the epidermal growth factor receptor to transform 3Y1 cells (20). We have also found that stimulation of PLD activity transforms the 3Y1c-Src cells.5 Because tamoxifen reduced the level of PKCδ in the 3Y1c-Src cells, we examined whether tamoxifen treatment elevated PLD activity in the 3Y1c-Src cells. As shown in Fig. 5, tamoxifen treatment led to an increase in PLD activity in 3Y1c-Src cells but not parental 3Y1 cells. Thus tamoxifen, similar to TPA and other negative regulators of PKCδ (10), elevates PLD activity. Because PLD activity is sufficient to transform 3Y1 cells overexpressing a tyrosine kinase (20), it is possible that the ability of tamoxifen to elevate PLD activity in the 3Y1c-Src cells is important for the transformed phenotypes observed.

**Discussion**

How tamoxifen stimulates the association between c-Src and PKCδ is not known. We demonstrated previously that association between v-Src and PKCδ depended upon an active Src kinase. Thus, it is likely that tamoxifen treatment leads to the activation of c-Src through a mechanism yet to be determined. Tamoxifen had no effect upon c-Src protein levels (data not shown). The effect may be specific for c-Src or non-receptor class tyrosine kinases because tamoxifen does not have the tumor-promoting effects seen in the 3Y1c-Src cells in 3Y1 cells that overexpress the epidermal growth factor receptor.5 Tamoxifen is known to have estrogen-mimetic effects in endometrial cells (21). However, the effects of tamoxifen observed here go beyond estrogen-mimetic effects, because estrogen did not cause the transformation-related phenotypes caused by tamoxifen.

Land et al. (19) described two oncogene complementation groups for the transformation of primary rodent cells. The essential features of this model have been verified in an updated version, which integrates the role of telomerase in the malignant transformation of human cells (22). According to this model, a cytoplasmic signaling oncogene, such as ras or src, cooperates with a nuclear oncogene, such as myc or SV-40 large T antigen, to cause transformation (19). Interest

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5 Unpublished results.
ingly, TPA was able to cooperate with the signaling, but not the nuclear oncogenes, to transform primary cells (23), indicating that TPA could accomplish the equivalent of Myc or SV-40 large T. This would indicate that tamoxifen facilitates passage through the G₁-S cell cycle checkpoint, because large T antigen exerts its effects by sequestering Rb and p53 (24).

The relatively rapid increase in endometrial cancers seen with tamoxifen treatment suggests that tamoxifen may have multiple carcinogenic effects. The estrogen mimetic effects of tamoxifen, which causes endometrial hyperplasia, most likely explain why endometrial cancers are seen first with prolonged tamoxifen treatment (21, 25). The tamoxifen metabolite 4-OH-tamoxifen, which has DNA-damaging capability (26), is present in the serum of patients taking tamoxifen (27) and could therefore also contribute to tumor progression. The stimulation of cell proliferation in the presence of DNA-damaging agents significantly increases mutation rates, and tumor progression is accelerated (28). Thus, the combination of the cell proliferation-inducing estrogen-mimetic effects of tamoxifen and DNA damage brought about by 4-OH-tamoxifen should make tamoxifen a potent carcinogen. However, cigarette smoke, which also contains both tumor-promoting and DNA-damaging agents (29), takes >20 years before significant increases in lung cancer are observed. Therefore, it is likely that there are other effects of tamoxifen that make it an even more potent carcinogen that induces endometrial cancers within 10 years. The TPA-mimetic properties reported here could be the additional carcinogenic effect responsible for the rapid tumor-producing ability of tamoxifen. Thus, tamoxifen may be a three-pronged carcinogen that produces estrogen-mimetic hyperplasia, DNA damage, and a TPA-mimetic down-regulation of the tumor-suppressing PKCδ. Although the effects of tamoxifen in this cell culture model may not reflect exactly the effects of tamoxifen in human endometrial cells, they do
provide evidence of a tumor-promoting effect for tamoxifen that is consistent with its apparent tumor-promoting effects in patients taking tamoxifen for extended periods. If true, even longer term tamoxifen treatment, which has been proposed as a preventative step for women deemed at risk for breast cancer, could ultimately result in a much higher incidence of endometrial cancers and perhaps other cancers later on. Although the benefits of tamoxifen treatment for 5 years clearly outweigh the risks of a small increase in endometrial tumors, longer term prophylactic treatment needs to be considered carefully.

Materials and Methods

Cell Lines and Culture Conditions. 3Y1 rat fibroblasts and 3Y1 cells overexpressing c-Src (3Y1c-Src cells, clone 4) were generated as described previously (9). Cells were maintained in DMEM supplemented with 10% bovine calf serum (HyClone). Anchorage-independent growth was assayed by suspending cells in soft agar as follows. Cells (1 \times 10^6) were suspended in top agar (DMEM, 20% calf serum, and 0.38% agar) and overlaid onto hardened bottom agar (DMEM, 20% calf serum, and 0.7% agar) as described previously (9). Colonies were counted 14 days later.

DNA Synthesis Assays. Cells were made quescent by growing to confluence and then placing in fresh medium containing 0.5% bovine calf serum for 36 h in 24-well tissue culture dishes. DNA synthesis was measured by a 1-h pulse with [3H]thymidine (1 \muCi/ml; 20 Ci/mmol). Cells were then collected, and trichloroacetic acid-precipitable counts were determined by scintillation counting as described previously (9).

PKC\alpha Kinase Assays. PKC\alpha activity was determined using a protein kinase assay kit from Calbiochem. Cells were prepared by washing with cold PBS (136 mM NaCl, 2.6 mM KCl, 1.4 mM KH\(_2\)PO\(_4\), and 4.2 mM Na\(_2\)HPO\(_4\), pH 7.4), scraping with a rubber policeman, suspension in kinase sample buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 5 mM EGTA, 50 mM 2-mercaptoethanol, 100 \muM Na-VO\(_4\), and protease inhibitor mixture], and then sonication for 30–60 s to disrupt cell membranes. The cell extracts were then clarified by centrifugation at 100,000 \times g for 1 h at 4°C. Equal amounts of protein were then subject to immunoprecipitation with an anti-PKC\alpha antibody (Santa Cruz Biotechnology). Immune complexes were recovered with protein A-agarose beads and subjected to the kinase assay according to the manufacturer’s instructions.

PLD Assays. Subconfluent 35-mm culture dishes were prelabeled for 4 h with [3H]myristate, 3 \muCi (40 Ci/mmol) in 3 ml of medium containing 0.5% newborn calf serum. PLD-catalyzed transphosphatidylation in the presence of 1% butanol was performed as described previously (9, 10, 20).

Western Blot Analysis. Extraction of proteins from cultured cells was performed as described previously (9). Equal amounts of protein were subjected to SDS-PAGE using an 8% acrylamide separating gel, transferred to nitrocellulose, and blocked overnight at 4°C with 5% nonfat dry milk isotonic PBS (136 mM NaCl, 2.6 mM KCl, 1.4 mM KH\(_2\)PO\(_4\), and 4.2 mM Na\(_2\)HPO\(_4\)). The nitrocellulose filters were washed three times for 5 min in PBS and then incubated with antibodies as described in the text. Depending upon the origin of the primary antibodies, either antimouse or antirabbit IgG was used for detection using the ECL system (Amersham).

References


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