Tamoxifen Attenuates Glucocorticoid Actions on Bone Formation in Vitro*

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ABSTRACT

Tamoxifen is a synthetic estrogen analog which may regulate osteogenesis in vitro by virtue of its antiglucocorticoid properties. We have examined tamoxifen regulation of glucocorticoid-induced osteogenesis in two different in vitro bone systems: the chicken periosteal osteogenesis model (CPO) and rat bone marrow stromal cells (RBMC). Hormone uptake studies were conducted with the osteosarcoma cell line, ROS 17/2.8. In the CPO model, alkaline phosphatase (AP) activity and collagen synthesis were stimulated by the glucocorticoid dexamethasone (Dex; 0.1 μM). These Dex-mediated effects were inhibited by increasing concentrations of tamoxifen (10–100 μM). Similarly, in the RBMC model, Dex-dependent (0.01 μM Dex) mineralized tissue formation and AP activity were blocked by tamoxifen (0.1 μM). Although tamoxifen inhibited Dex-mediated increases of AP activity in ROS 17/2.8 cells, it did not inhibit uptake of 3H-Dex or of 3H-estrogen. Northern analyses showed that tamoxifen did not affect messenger RNAs (mRNAs) for AP. Tamoxifen did seem to reduce mRNA for collagen type I, but not bone sialoprotein, osteopontin, and osteocalcin. Dex-induced increases for all proteins mRNAs in the RBMC model were not reduced by tamoxifen. Similarly, tamoxifen had no effects on cellular proliferation. We conclude that tamoxifen has no direct effect on gene expression of bone-related proteins of osteoblastic cells. Further, in the ROS 17/2.8 cell line, the antiglucocorticoid properties of tamoxifen do not appear to be mediated through either Dex or estrogen receptors. (Endocrinology 138: 3269–3275, 1997)

Tamoxifen is a synthetic estrogen analog with pronounced antiestrogenic effects (1) and milder estrogenic activities (2). This drug has been shown to be an effective agent for the treatment of advanced breast carcinoma (3) and for prophylaxis against recurrence (4). It is believed that tamoxifen acts by binding to estrogen receptors in estrogen receptor rich breast carcinoma cells (5) and by inhibiting their growth (6). Although tamoxifen is indicated for use in estrogen receptor positive cells, it may also act on estrogen receptor negative cells, suggesting an alternative mode of action that is independent of estrogen receptor binding (7). Indeed, tamoxifen may be used in treatment of other malignancies that are neither estrogen receptor positive nor related to breast cancer (8, 9).

Similarly, tamoxifen induces unanticipated effects on bone metabolism. As tamoxifen is an antiestrogen, it might be expected to cause osteoporosis as is seen in postmenopausal females (10, 11). However, tamoxifen reduces bone loss in these patients (12), possibly because it can act as an estrogen agonist (1, 13). Currently, the biological mechanisms underlying the bone-sparing effects of tamoxifen are unclear (1). Although tamoxifen may interact directly with estrogen receptors in bone and mimic estrogen effects, the direct actions of tamoxifen and estrogen on bone cells are not well understood (1, 4, 14). It is uncertain whether the effects of tamoxifen on bone are simply those of a sex-steroid, mediated through its mild estrogenic activities. Notably, tamoxifen abrogates the effects of exogenous glucocorticoids in vivo (15), antiinflammatory agents that are potent inducers of bone loss in vivo (16). As the glucocorticoid and progesterone antagonist RU38486 exerts protective effects on bone metabolism (17), we have examined whether tamoxifen might exhibit anti-glucocorticoid properties in osteogenic cells. In this study, we demonstrate that tamoxifen attenuates most glucocorticoid effects on osteogenesis in vitro.

Materials and Methods

Culture systems

Two well characterized models for bone formation in vitro were used: the chicken periosteal osteogenesis (CPO) model (18) and the rat bone marrow stromal culture (RBMC) system (19). Although these models are derived from different species, are reliant upon cells from different sources (CPO, mesenchymal; RBMC, marrow) and from different stages of development, the two models are complementary to one another and permit the assessment of factors that modulate bone formation. Various aspects of bone formation can be analyzed using these systems including osteogenic cell proliferation (e.g. 3H-thymidine incorporation), differentiation (alkaline phosphatase activity), bone matrix formation (type I collagen synthesis), and mineralization (calcium accumulation or alizarin red staining). To analyze receptor binding, a well established clonal osteoblastic cell line, ROS 17/2.8 was used (20).

The CPO model has been described previously (18, 21). Over a 6-day culture period, morphologically distinct bone is formed within the folded periosteal tissues in the presence of 10 mM β-glycerophosphate (GP). This bone is essentially indistinguishable from that formed in vivo (18).

For the RBMC model, femoral bones derived from adult male Wistar rats were removed under aseptic conditions, cleaned of adherent soft tissues, and washed in antibiotics. Male rats were selected to facilitate...
analysis of tamoxifen and Dex interactions by reducing the confounding effects of functional estrogen receptors (22) as male rats should have few of these. The dye-binding assays were performed and the marrow contents were flushed out with 10 ml of culture medium followed by repeated passage of the harvested cells through a 20-gauge needle. The culture conditions and staining for bone nodule formation have been described in detail elsewhere (19, 21). This method of analysis reflects actual nodule formation and not just mineralization. Furthermore, this can be confirmed using phase contrast microscopy.

ROS 17/2.8 cells were cultured in medium containing DMEM supplemented with 10% FCS and antibiotics at a cell-density of 1 × 10^6 cells/cm^2 in T-25 tissue culture flasks (Falcon, Lincoln Park, NJ). Cultures were incubated under the atmospheric and temperature conditions described above.

Tamoxifen treatment

Cultures were treated with Dex and tamoxifen singly or in combination up to the end of their respective culture periods. Control cultures were treated only with vehicle (ethanol). Both CPO and RBMC cultures were treated with Dex at a concentration of 0.1 and 0.01 μM respectively, concentrations shown to up-regulate osteogenesis in the CPO model (21, 23).

Outcome measures

Chicken periosteal osteogenesis model. Bone matrix formation in the CPO system was assessed by analysis of either total or newly synthesized radiolabeled type I collagen. Collagen synthesized on days 4–6 was radiolabeled by the addition of 14C glycine (Amersham, 10 μCi/ml, 59 mCi/mmol) to the culture medium. At the end of the culture period (day 6), cultures were frozen at −20 °C, and analysis of nascent type I collagen was subsequently carried out as described previously (21).

In addition to assessment of bone matrix synthesis, a panel of biochemical assays, including alkaline phosphatase activity (cellular differentiation), soluble protein content (culture size), calcium and phosphate levels (mineralization), and 3H-thymidine incorporation (proliferation) was established to measure osteodifferentiation and osteogenesis in the cultures. These methods have already been described in detail (21, 23).

Rat bone marrow culture system. Osteogenesis was assessed in the RBMC model using alizarin red-S to stain for mineralized bone nodules. Cells that were grown for 12 days in 96-well plates were fixed with 10% neutral-buffered formalin as described earlier (24). In this case, the cultures were grown in 96-well plates, and mineralized (alizarin red-stained) tissue was quantified by using a Titertek plate reader to determine the optical density at 592 nm. As noted above, nodule formation was also screened by phase-contrast microscopy. Cellular proliferation, as based on 3H-thymidine incorporation was also assessed.

To assess the effects of tamoxifen and its putative interactions with Dex on gene expression, Northern blot analysis was performed on RBMC cultures. Full-length complementary DNA probes for alkaline phosphatase, osteopontin, osteocalcin, type I collagen, and bone sialoprotein were used. RNA was extracted from RBMC cultures using routine acid guanidium thiocyanate methods (25). Northern analysis was then conducted on the prepared RNA sample as described (26). Signals were quantified using a Molecular Dynamics Phosphoimaging System and MD Imagequant Software version 3.3. Hybridization to 18S RNA was used to correct for unequal loading between lanes.

Results

Chicken periosteal osteogenesis model

Dex treatment increased alkaline phosphatase activity greater than 4-fold (P < 0.05). This increase was reversed either completely (Fig. 1A) in a dose response from 10–100 μM tamoxifen or at least by 50% (Fig. 1B) in the presence of 25 μM of the drug depending on the developmental phase of the culture. Reversal of Dex effects on alkaline phosphatase activity was observed to various degrees regardless of when Dex-treated cultures were exposed to tamoxifen (Fig. 1B). Consistent with the alkaline phosphatase data, newly synthesized collagen production was increased in the presence of Dex, and this effect was blocked completely by tamoxifen (Fig. 1C). Notably, tamoxifen did not affect collagen synthesis when added alone, whereas there was an observable effect, at 25 μM, on alkaline phosphatase activity. As shown previously (21), Dex reduced calcium incorporation, as did tamoxifen (Fig. 1D). When Dex and tamoxifen were added concurrently, their inhibitory effects on mineralization were attenuated. Histological evaluation of drug-treated CPO cultures did not suggest gross microscopic evidence for toxicity of tamoxifen (25 μM) or Dex (0.1 μM) (Fig. 2). However, 100 μM tamoxifen did appear to be toxic. Tamoxifen did not alter Dex-mediated effects on 3H-thymidine incorporation.

Rat bone marrow cells

In the absence of Dex, RBMC cultures did not form mineralized bone nodules as shown previously (21). Dex-induced bone nodule formation was inhibited by increasing concentrations of tamoxifen, almost to zero as shown in cultures without Dex (Fig. 3). When added singly, tamoxifen did not induce nodule formation and did not affect alkaline phosphatase activity. Northern analysis (Fig. 4a) showed that Dex induced greater than 3-fold increases of messenger RNA (mRNA) for bone sialoprotein, osteopontin, osteocalcin, alkaline phosphatase, and type I collagen vs. control (when normalized for 18S RNA) (Fig. 4b). Although tamoxifen clearly inhibited Dex-induced increases in bone nodule formation, it did not reduce Dex-induced increases in mRNA for type I collagen or other matrix or mineral-associated proteins such as osteopontin, osteocalcin, and bone sialoprotein (Fig. 4). Similarly, although tamoxifen markedly reversed Dex-induced increases in alkaline phosphatase activity, there was
only slight reduction in mRNA. When added singly, tamoxifen had no measurable effects on mRNA levels for any of the above described proteins with the notable exception of COL1. Finally, tamoxifen had no measurable effects on cellular proliferation, as based on 3H-thymidine incorporation, either alone or in combination with Dex (not shown).

ROS 17/2.8 cells

As shown by others (20), Dex increased alkaline phosphatase activity by 50% \( (P < 0.05) \) compared with vehicle control (Fig. 5). These Dex-induced increases of alkaline phosphatase activity were reversed in a dose-dependent fashion and by as little as 5 \( \mu \)M tamoxifen, confirming the antiglucocorticoid effects described for the other models.

Uptake of 3H-Dex in ROS 17/2.8 cells was competitively inhibited by unlabeled Dex, indicating the presence of glucocorticoid receptors in this cell line (21, 28). Uptake of 3H-Dex was not reduced in the presence of increasing concentrations of either tamoxifen or unlabeled estrogen (Fig. 6a). As the tamoxifen effects might be mediated indirectly via interaction with an estrogen receptor, 3H-estrogen uptake was investigated, but there was no evidence for estrogen receptors in these cells (Fig. 6b). Moreover, even basal uptake of radiolabeled estrogen was not affected by unlabeled Dex, tamoxifen, or estrogen (Fig. 6b). On the basis of flow cytometric data, tamoxifen (1 \( \mu \)M, 5 \( \mu \)M, or 10 \( \mu \)M) either alone or in combination with Dex did not appear to have marked effects on the percentage cells in S-Phase (Dex alone: 16%; Dex-tamoxifen: 17%; No Drug: 19%, tamoxifen alone: 21%), suggesting minimal effects on proliferation.

**Discussion**

Using a variety of bone cell and bone formation systems, we have shown that tamoxifen interferes with the action of glucocorticoids on osteogenic cells. At least in ROS 17/2.8 cells, these effects do not appear to be mediated through glucocorticoid or estrogen receptors, but further studies are needed to demonstrate this in the other models. However, as these models (CPO, RBMC) have much more heterogeneous cell populations, radiolabeled hormone uptake studies would not be as reliable as in the more homogeneous ROS 17/2.8 cell line. Indeed, pilot studies in our laboratory indicate that although the CPO and RBMC cultures are highly responsive to Dex, it is very difficult using the assays described here, to identify even the Dex receptors. Accordingly, more sophisticated cytosol binding assays or RT-PCR approaches, will be required in those models. Thus, it must be emphasized that the receptor-binding data shown here are predominantly applicable to ROS 17/2.8 cells. As there were consistent changes in the levels of alkaline phosphatase and collagen synthesis without alterations in mRNAs for these genes, the antiglucocorticoid effects of tamoxifen may in-
volve posttranscriptional events. However, further assessment of other proteins synthesized by bone, in addition to those already shown here, as well as studies focused on posttranscriptional events are essential.

**Antiglucocorticoid effect**

Drugs with antiglucocorticoid properties such as RU38486 (17) may protect against the development of hypogonadal osteopenia. Because tamoxifen demonstrates protective effects on bone in hypogonadal conditions (29), we explored the notion that it may also possess antiglucocorticoid properties. Our data show that tamoxifen inhibited Dex-mediated effects in all of the models evaluated. As the antiglucocorticoid effects of tamoxifen were shown in an avian (CPO) and mammalian (RBMC) bone formation model as well as a mammalian bone cell line (ROS 17/2.8), this is evidently not a species or model-specific phenomenon. However, there is evidence in a human cell line suggesting an opposite effect for mineralization (30) but, notably, this was shown in a tumour cell line. Mineralization in such cell lines may not necessarily represent actual bone formation but rather precipitation of hydroxyapatite, and so this could explain such differences. The previous finding that tamoxifen possesses antiglucocorticoid effects in vivo supports our contention that the results obtained here are not culture artifacts (15). Further, other findings in this laboratory show that tamoxifen inhibits Dex-mediated reduction of longitudinal bone growth in a piglet model. We conclude that tamoxifen possesses glucocorticoid-inhibitory properties that are important in bone metabolism. In most cases, this effect is only mediated in the presence of a glucocorticoid as tamoxifen by itself had few effects at the doses used here. However, in some cases tamoxifen inhibited some parameters of bone formation on its own (e.g., alkaline phosphatase at 25 μM). Thus, some of the “antiglucocorticoid” properties of tamoxifen could be related to an opposing effect rather than antiglucocorticoid actions per se. The effect of 100 μM tamoxifen was related to toxicity as judged by histological assessment.

**Tamoxifen effect on Dex or estrogen uptake**

To elucidate the mechanisms underlying this phenomenon, we determined whether tamoxifen inhibited cellular uptake of Dex as an indirect measure of binding of Dex to its cognate receptor. ROS 17/2.8 cells were used as a model because AP activity in this cell line was modulated by either Dex or tamoxifen, similar to the other models used here. Moreover, the ROS 17/2.8 cell line is more homogeneous than either the CPO or RBMC models, and so it was thought that the data would be simpler to interpret. Thus, as noted above, the receptor binding data apply largely, but perhaps

![Fig. 2. Histological features of CPO cultures. A, Culture incubated without Dex or tamoxifen. B, Culture treated with Dex alone. C, Tamoxifen alone (25 μM). D, Culture was grown in presence of Dex and 25 μM tamoxifen. All cultures were incubated with various drugs or hormones from the outset and stopped at day 6. For all treatments, there is a well defined mineralized area (M) bordered by a mineralization front, the presence of osteocytes (→) osteoblasts (←), an area of osteoid (O) and an outer fibrous layer (f). Magnification, 640×. The bar in each section is 20 μM.](https://academic.oup.com/endo/article-abstract/138/8/3269/2987794)
not solely, to the ROS 17/2.8 cell line that is, nonetheless, osteoblastic in nature. \(^{3}\)H-Dex uptake was inhibited in a competitive fashion by unlabeled Dex, indicating the presence of receptor-mediated binding. In contrast, tamoxifen exerted no such effect. We suggest that the abrogation of Dex effects by tamoxifen was not related to inhibition of Dex uptake by target cells and, by extension, Dex binding to its cognate receptor, as shown in previous studies (21, 28). We also explored the notion that tamoxifen mediates its effects through the estrogen receptor, but our data indicate that the ROS 17/2.8 cell line takes up little or no estrogen and any basal uptake of estrogen that is present is neither competed down by unlabeled estrogen nor by tamoxifen. As demonstrated in other cell types (7, 9), this finding suggests that the effects mediated by tamoxifen are not mediated through either estrogen or Dex receptors, at least in ROS 17/2.8 cells. Moreover, a previous investigation using another bone cell culture system (HOS TE-85) showed that tamoxifen-induced increases in mineralization were not mediated through estrogen receptor binding or alteration of estrogen receptor response element activation (30), consistent with the data reported here. Nonetheless, it must be emphasized here that receptor binding studies were not undertaken in the other two models for reasons alluded to above, and so arguments related to receptor binding must be restricted to the findings reported in the ROS 17/2.8 cell line used here. Further investigations (using alternate methods alluded to above) focused on the estrogen receptor levels in CPO and RBMC model are being pursued at this time.

FIG. 3. Dose response of tamoxifen, (with and without Dex) in the RBMC. In the presence of Dex, mineralized nodules were formed by day 10. Tamoxifen reverses this effect with almost complete abrogation of nodule formation at 0.1 \(\mu M\). Tamoxifen alone does not induce nodule formation. There were no mineralized nodules in the absence of Dex.

FIG. 4. A, Northern hybridization bands for bone sialoprotein (BSP), osteopontin (OPN), osteocalcin (OC), alkaline phosphatase (AP), and type I collagen (Coll I) are shown here along with bands for 18S ribosomal RNA (rRNA). Although there may appear to be some differences in band intensities between Dex and Dex plus tamoxifen groups, this was not shown following normalization against 18S rRNA as shown in Fig. 4B. B, Relative levels of mRNA in 12-day-old RBMC for collagen type 1 (COLL1), osteocalcin (OC), osteopontin (OPN), bone sialoprotein (BSP), and alkaline phosphatase (AP). The levels in the Dex-treated cultures were taken as 100%, and all values were normalized against 18S rRNA. Tamoxifen (0.1 \(\mu M\)) alone did not affect mRNA levels compared with control except for COLL1. Dex increased mRNA levels for all proteins measured here. Tamoxifen did not affect Dex-induced increases in mRNA levels for osteopontin, osteocalcin, or type 1 collagen and showed only very slight attenuation of mRNA for alkaline phosphatase.
Tamoxifen effects on expression of mRNAs for bone proteins

Because data obtained in the ROS 17/2.8 cells suggested that the glucocorticoid-inhibitory effects of tamoxifen might not be mediated through glucocorticoid or estrogen receptors, we asked if mRNA levels for various proteins known to be up-regulated by Dex would be affected by tamoxifen. The data show that tamoxifen did not inhibit up-regulation of mRNA for any of the proteins studied. However, tamoxifen completely abrogated Dex-induced formation of bone nodules. Accordingly, concomitant Dex-induced increases in collagen production and alkaline phosphatase activity were also inhibited, whereas mRNA for those proteins (as well as the others) were not. These findings could suggest that tamoxifen inhibits Dex effects through as yet unidentified posttranscriptional mechanisms, results that are not surprising in view of the receptor binding results discussed above. This apparent disparity between the mRNA data and the protein synthesis data could also be attributable to cellular heterogeneity in the RBMC model. Notably, we did not measure production of all of the noncollagenous proteins. However, preliminary analysis of 35S-methionine radiolabeled noncollagenous proteins extracted from the cell layer in RBMC cultures suggests at least a 4-fold reduction in tamoxifen plus Dex treated cultures as compared with Dex alone. Furthermore, preliminary immunoprecipitation studies for bone sialoprotein suggest similar results. Confirmatory immunoprecipitation studies are now underway for bone sialoprotein and the other proteins for which message data were obtained.

Although the mechanisms underlying the effects of tamoxifen on bone cells are not clear, there is evidence that tamoxifen may interact with cell membrane receptors for insulin-like growth factor (IGF) (31) or may regulate the production of IGF binding protein (32) or may alter secretion of transforming growth factor-β (30), all of which are known to affect bone metabolism. There is also evidence that tamoxifen may interact with antioestrogen binding sites, which are distinct from estrogen receptors (33). Finally, although mRNA for bone proteins was not altered by tamoxifen, this does not preclude the probability that other genes such as c-myc (7) could have been regulated in bone, and this requires more study.

Cellular proliferation

The data presented here suggest that glucocorticoid-mediated effects on cellular proliferation are not altered by tamoxifen. That this was observed in all three models used suggests that this is not a species or model specific phenomenon. Moreover, inasmuch as 3H-thymidine uptake is not the
only way to assess cell proliferation, we used an alternate method in the ROS 17/2.8 cells, flow cytometry. Similar findings (i.e. no or minimal effect) were obtained that would further tend to confirm the notion that tamoxifen-mediated attenuation of Dex effects is not accomplished through alterations in proliferation. Thus, it appears that tamoxifen inhibits the cell’s ability to organize into nodules but may not inhibit their proliferation. This raises another interesting issue pertaining to the ability of tamoxifen to block the effects of Dex in that the former only attenuates the actions of Dex for certain parameters (e.g. alkaline phosphatase, collagen synthesis) but not others (e.g. mRNA levels, proliferation). The fact that the antiglucocorticoid effects are so specific suggests, but certainly does not prove, that these effects are probably independent of Dex receptors. In this regard, it might be expected that if tamoxifen interfered with the ability of Dex to interact with its cognate receptor, its antiglucocorticoid actions would be more global in nature, but such was not the case. Moreover, and as discussed above, some of the effects of tamoxifen could in fact be pharmacological but opposite to those of Dex. This may be the case for the effects on alkaline phosphate activity in which tamoxifen actions, being opposite to those of Dex, essentially reverse Dex-mediated increases in that enzyme’s levels.

Although our findings do not explain how tamoxifen prevents postmenopausal bone loss, it is known that sex steroids inhibit glucocorticoid actions (34, 35). Moreover, it must also be recognized that in addition to the effects of tamoxifen and glucocorticoids noted in this investigation, the skeletal effects of these agents may also be mediated by systemic modifications in hormonal regulation of calcium homeostasis. However, as shown here for tamoxifen and previously for RU38486 (21), perhaps the sex steroids or their “inactive” analogues inhibit or antagonize the effects of endogenous glucocorticoids. Consequently, sex steroids may protect against postmenopausal osteoporosis by minimizing the osteoporosis-inducing effects of endogenous corticosteroids. Thus, other agents that can attenuate glucocorticoid actions on bone such as the transforming growth factor-β binding protein fetuin (24) might have similar properties.

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