Efficacy of LGD1069 (Targretin), a Retinoid X Receptor-Selective Ligand, for Treatment of Uterine Leiomyoma

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ABSTRACT

The conventional treatment of uterine leiomyomas, or fibroids, with gonadotropin-releasing hormone (GnRH) agonists is often associated with serious side effects, necessitating short-term, palliative use of this therapy. Therefore, we examined a retinoid X receptor (RXR)-selective ligand, LGD1069, as a possible treatment for leiomyoma. LGD1069 has demonstrated efficacy as a chemopreventive agent in the N-nitroso-N-methyleurea (NMU)-induced rat mammary carcinoma model and is a therapeutic agent in several epithelial tumor models. Previous studies have shown that it has both antitumor effects and antiestrogenic activity in the rat uterus, suggesting the potential utility of this agent for treatment of hormonally dependent uterine fibroids. The expression of retinoid receptors in tumors and cell lines derived from leiomyomas arising in the Eker rat was confirmed by Northern analysis. After treatment for 4 months with LGD1069, the number of grossly observable tumors was substantially reduced although the total incidence of tumors, including microscopic lesions, remained unaffected, suggesting an effect of the compound on tumor growth kinetics rather than on tumor initiation. Analysis of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining and determination of 5-bromo-2-deoxyuridine (BrdU) incorporation indicated that the reduction in grossly observable tumors that occurred in treated animals was mediated by a significant increase in the level of apoptosis rather than a decrease in cell proliferation. These results suggest that LGD1069 may be an effective therapeutic agent for uterine leiomyoma that may inhibit tumor growth and, consequently, alleviate the symptoms associated with this disease.

Leiomyomas, or fibroids, are the most common gynecological neoplasm in women, with a reported incidence of 50 to 70% (Cramer and Patel, 1990). Although generally considered benign, this tumor has been associated with spontaneous abortion, infertility, and menorrhagia, and is the leading cause for premenopausal hysterectomy in the United States (Buttram and Reiter, 1981). Nonsurgical treatment of leiomyomas with gonadotropin-releasing hormone (GnRH) agonists that inhibit ovarian steroid production has proven successful in inducing tumor regression (Verkauf, 1993). However, prolonged use can lead to bone loss, increased blood lipid levels, and other side effects typical of estrogen abrogation, necessitating GnRH therapy to be temporary (Johansen et al., 1988; Dawood et al., 1989). Cessation of treatment often results in a rapid regrowth of the tumor (Friedman et al., 1989). Therefore, novel approaches that inhibit leiomyoma progression would have therapeutic value as an alternative to surgery and to palliative agents with adverse side effects.

One class of compounds that has shown efficacy in the clinical treatment of a variety of human malignancies is the retinoids (Huang et al., 1988; Lippman et al., 1993). All-trans-retinoic acid (ATRA) is an endogenous hormone, generated from vitamin A, that regulates a variety of physiological processes, including cell growth and differentiation (Lotan, 1980). The effects of ATRA are mediated through the retinoic acid receptor (RAR), a member of the intracellular receptor superfamily. Members of the intracellular receptor superfamily include the retinoic acid receptor (RAR), a member of the intracellular receptor superfamily. Members of the intracellular receptor superfamily are ligand-dependent transcription factors that act through regulation of gene expression in target tissues (Mangelsdorf et al., 1990).

In addition to the RARs, another class of retinoid receptors has been identified called the retinoid X receptors (RXRs) (Mangelsdorf et al., 1990). Although ATRA binds only to the retinoic acid receptor superfamily, RXRs bind to both RARs andRXRs.

Abbreviations:
ATRA, all-trans-retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; 9-cis-RA, 9-cis-retinoic acid; BrdU, 5-bromo-2-deoxyuridine; NBF, neutral-buffered formalin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; HPF, high-power field; CEH, cystic endometrial hyperplasia; NMU, N-nitroso-N-methyleurea; GnRH, gonadotropin-releasing hormone.
RAR family of retinoid receptors, the endogenous ligand 9-cis-retinoic acid (9-cis-RA) binds to and activates both the RAR and RXR families of receptors (Heyman et al., 1992; Levin et al., 1992; Allenby et al., 1993). The RXRs not only function as the effector proteins for 9-cis-RA but also as obligate heterodimeric partners for other members of the intracellular receptor superfamily, including those binding to retinoic acid, thyroid hormone, peroxisome proliferator-activator, vitamin D, and others whose ligands have yet to be identified (orphan receptors) (Yu et al., 1991; Kliewer et al., 1992a,b; Leid et al., 1992).

Reports indicate that 9-cis-RA, a pan agonist, was better able to prevent N-nitroso-N-methylurea (NMU)-induced mammary tumors and progression of xenografts in nude mice than was ATRA, an RAR-specific ligand (Anzano et al., 1994; Gottardis et al., 1996a). These and other results suggest that the improved antitumorigenicity of 9-cis-RA may be due in part to the induction of the RXR pathway, suggesting an improved therapeutic utility of molecules that bind to and stimulate transactivation selectively through RXRs. One of the first RXR-specific, high-affinity ligands to be synthesized was LGD1069 (Targretin) (Boehm et al., 1994).

Previous studies with LGD1069 have demonstrated that it is an effective inhibitor of estrogen-mediated responses both in vitro and in vivo. LGD1069 inhibited estrogen-stimulated uterine wet weight increases in the 3-day immature rat assay (Gottardis et al., 1996a) and inhibited estrogen-dependent proliferation of breast cancer cell lines (Fitzgerald et al., 1997). LGD1069 has also been shown to be a highly effective chemopreventive agent in the NMU-induced hormone-dependent rat mammary carcinoma model, and was as efficacious as tamoxifen at inhibiting the development of mammary tumors (Gottardis et al., 1996a). LGD1069 also inhibited the growth of carcinogen-induced mammary neoplasms and induced complete regression of 72% of the primary tumors (Bischoff et al., 1998).

The mechanism for the antiestrogenic effect of LGD1069 has yet to be elucidated; however, Joyeux et al. (1996) have reported that the antiestrogenicity may not be merely the result of the ability of both estrogen and retinoid receptors to bind to estrogen response elements (Joyeux et al., 1996). Rather, this effect may be due to competition for shared coactivators that enhance the interaction of the receptors with transcriptional machinery. Competition for limited auxiliary proteins by ligand-activated receptors could inhibit estrogen receptor transactivation through a process known as squelching.

As a result of these studies and the success of LGD1069 at inhibiting tumors in the rat mammary tumor model, LGD1069 was evaluated for efficacy in inhibiting the development of uterine leiomyoma in the Eker rat, a recently developed animal model for this disease (Everitt et al., 1995). We report that LGD1069 reduced the incidence of grossly observable tumors in these rats, which was mediated by an increase in the apoptotic index in treated tumors.

Materials and Methods

Cell Culture for Northern Analysis. Five cell lines (ELT-3, ELT-4, ELT-6, ELT-9, and ELT-10), derived from uterine leiomyomas from Eker rats, were examined by Northern analysis for the expression of RAR and RXR isozymes. One additional cell line, ELT-5B, was derived from a leiomyosarcoma. Each cell line was maintained and propagated in culture on plastic culture dishes (Corning, Cornings, NY) in DMEM medium containing 10% fetal bovine serum (HyClone Laboratories, Logan, UT) as previously described (Howe et al., 1995). Washed cells were lysed in 4 M guanidine thiocyanate solution and total RNA was isolated using a cesium chloride gradient as described by Chirgwin et al. (1979). Poly(A)+ RNA was then separated by oligo(dT) cellulose affinity chromatography.

Tumors for Northern Analysis. Leiomyoma samples from two different 16-month-old Eker rats from other studies ongoing concurrently were also examined by Northern analysis for the expression of retinoid receptors. The TAM2 tumor was isolated from a tamoxifen-exposed rat and the C10 tumor was harvested from an untreated control animal.

Northern Analysis. Northern blots were prepared by electrophoretically separating 5 μg of poly(A)+ RNA on a 1% agarose gels and transferring to nylon membranes as previously described (Sambrook et al., 1989). The cDNA probes corresponding to RARα (Giguere et al., 1987), RARβ (de The et al., 1987), RARγ (Krusto et al., 1989), RXRα (Mangelsdorf et al., 1990), RXRβ (Fleischhauer et al., 1992), RXRγ (W. W. Lamp, unpublished data), and GAPDH (Ambion, Inc., Austin, Texas) were labeled with [32P]dCTP using Ready-to-Go labeling beads (Pharmacia, Piscataway, NJ). Northern blots were hybridized and washed under high-stringency conditions as previously described (Church and Gilbert, 1984).

Animals. Female rats (12–14 months), heterozygous for the Eker [TSC-2 (tuberous sclerosis complex-2)] mutation, were bred at Science Park (Smithville, TX) and transferred to Ligand Pharmaceuticals (La Jolla, CA) for the in vivo study where they were housed in a United States Department of Agriculture-registered facility in accordance with National Institutes of Health guidelines for the care and use of animals. The rats were housed under quarantine, with food (Harlan Teklad LM48507012; Harlan Breeders, Indianapolis, IN) and acidified water provided ad libitum. The tumor incidence, 33% in control Eker rats in this study, was lower than historical incidence data reported in other studies using this animal model (~62%) (Everett et al., 1995). Although the reasons for this are not clear, it may be attributable to transport stress associated with the shipment of rats from Texas to California.

Experimental Design. Starting at 12 months of age, Eker rats received 100 mg/kg (p.o.) LGD1069 daily (including weekends) for a 4-month period. The plasma concentrations achieved by daily dosing with 100 mg/kg p.o. are in the micromolar range (Gottardis et al., 1996); however, the dose used in our studies was based on previous work indicating that it was fully efficacious and nontoxic. Control animals (n = 24) did not receive LGD1069 and were housed and fed identically during the dosing period. After 4 months of dosing with LGD1069 (treated group) or when control animals reached 16 months of age, animals were sacrificed by CO2 asphyxiation as approved by institutional protocols, and the incidence and location of grossly visible uterine leiomyomas were recorded at necropsy.

Tissue Collection/Processing. Two hours before sacrifice animals were injected (i.p.) with a 20 μg/ml stock solution of 5-bromo-2-deoxyuridine (BrdU, 100 mg/kg final concentration). Uteri and vaginas were collected and fixed in 10% neutral buffered formalin (NBF) and stored for at least 24 h. Tumors, when present, were collected and portions of the samples were frozen in liquid nitrogen or fixed in 10% NBF. Collected tissue samples fixed in 10% NBF were embedded in paraffin according to standard protocols. Sections from all tissues collected were stained with H&E. Proliferation in treated and control tumors was determined by immunohistochemical localization of incorporated BrdU. The Apoptag apoptosis detection kit (Intergen, Purchase, NY), using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL), was employed to identify apoptotic cells in the tumors.

Analysis of BrdU and TUNEL Staining. For each tumor, 10 high-power fields (HPF, 400×) were counted and the level of proliferation and apoptosis was determined as the average number of...
BrdU- and TUNEL-positive cells/HPF, respectively. Values were normalized to account for differences in the total number of cells in control tumors and treated tumors. Statistical analysis of BrdU and TUNEL counts was determined by the two-tailed Student’s t test using Statview, version 5.0 (SAS Institute Inc., Cary, NC).

Results

Determination of Receptor Profile in Tumors and Cell Lines. Before initiating the in vivo therapeutic study, the presence of retinoid receptors in tumors arising in the Eker rat model was investigated to confirm the presence of the receptors for targeting with LGD1069. Primary tumors and tumor-derived cell lines were analyzed for receptor expression by Northern analysis. Both primary tumors and tumor-derived cell lines expressed the receptors RARα, RARγ, RXRα, and RXRγ (Fig. 1), but transcripts for RARβ and RXRβ were not present in detectable levels. Interestingly, in addition to the expected 2.89-kb mRNA transcript for RXRβ, additional transcripts of 4.1 kb (tumors and cell lines) and 1.74 kb (TAM2) were observed. The identity and importance of these additional transcripts is not known at this time.

Tumor Development. To determine the impact of LGD1069 on tumor development, 12-month-old female Eker rats were treated with compound daily for 4 months. In the control group, tumors were detected in 8 of 24 animals, with one animal having two large tumors. Of the nine total tumors, eight (88.8%) were observed grossly at necropsy, whereas one lesion was detected only by detailed microscopic analysis of uterine sections. In contrast, in LGD1069-treated animals the number of large, grossly observable tumors was significantly decreased \( P < .05 \) compared with controls (Fig. 2). In LGD1069-treated rats, a total of 13 tumors was observed in 38 animals, but few of the lesions (4 of 13 = 31%) were grossly visible at necropsy, whereas the majority (9 of 13 = 69%) were only detected microscopically during histological evaluation of the uter. Thus, the proportion of macroscopic tumors was shifted from \( > 88 \) to \( 31\% \) by LGD1069 treatment, although the overall tumor incidence of \( 33\% \) in control and \( 32\% \) (12 of 38) in treated animals was not significantly different. Although macroscopic lesions were reduced in LGD1069-treated rats, the distribution of histological subtypes (typical, epitheliod, or mixed) (Everitt et al., 1995) in observed lesions (macroscopic and microscopic) was not significantly different between treated and control animals.

Impact of LGD1069 on Endometrium and Vagina. The effect of LGD1069 on normal uterine endometrium and vaginal epithelium was assessed histologically in formalin-fixed sections stained with H&E. Overall, no major differences in histological appearance or presence of pathology were observed between control and LGD1069-treated tissues. Many uteri from both treated and control animals had areas of pronounced cystic endometrial hyperplasia (CEH). In 63% (24 of 38) of the treated animals and 50% (12 of 24) of the control animals, there was evidence of this background lesion, infrequently accompanied a highly dilated lumen (11% of the cases of CEH). The remaining animals had either a predominantly columnar endometrial epithelium, as seen in 13% (5 of 38) of the treated and 8% (2 of 24) of the controls, or a quiescent cuboidal epithelium found in 27% (8 of 38) of the treated and 42% (10 of 24) of the controls. Vaginae from both groups of animals were mucified; 79% (19 of 24) of vaginae from control animals and 83% (29 of 35) of vaginae from treated animals displayed mucification. However, in treated animals, 74% (26 of 35) of the vaginae had vacuolated cells, whereas only 13% (3 of 24) of the vagina from control animals had cells with vacuolization.

A apoptotic and Proliferative Indices. Apoptotic rates, as determined by the number of TUNEL-positive cells, were significantly higher in treated tumors \( (0.75 \pm 0.24 \text{TUNEL-positive cells/HPF}) \) compared with control tumors \( (0.12 \pm 0.03 \text{TUNEL-positive cells/HPF}) \), indicating that chronic (4-month) treatment with LGD1069 was associated with apoptosis in leiomyomas \( (P = .05) \) (Table 1; Fig. 3). In contrast, levels of proliferation, as determined by BrdU incorporation, were not significantly
TABLE 1
Summary of tumors, TUNEL, and BrdU incorporation

<table>
<thead>
<tr>
<th>Tumor Size</th>
<th>Number of Tumors</th>
<th>Control Animals (24)</th>
<th>Treated Animals (58)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macroscopic tumors (observed at necropsy)</td>
<td>8</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Microscopic tumors (not observed at necropsy)</td>
<td>1</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Total number of tumors</td>
<td>9</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>BrdU-positive cells (HPF)</td>
<td>0.12 ± 0.03</td>
<td>0.75 ± 0.24*</td>
<td></td>
</tr>
<tr>
<td>TUNEL-positive cells (HPF)</td>
<td>1.06 ± 0.315</td>
<td>4.13 ± 1.12</td>
<td></td>
</tr>
</tbody>
</table>

* $P < 0.05$ versus control tumors. A two-tailed Student's $t$ test was used to determine significance.

Discussion

Our studies determined that both RARs and RXRs are expressed in rat leiomyomas, indicating that these tumors have the potential to be responsive to retinoid treatment. Indeed, it has also been reported that human leiomyoma cells in culture express RAR and RXR receptors and were responsive to treatment with all-trans-retinoic acid (Boettger-Tong et al., 1997). In addition, Boettger-Tong et al. (1997) have demonstrated that cultured leiomyoma cells respond to retinoids and that ATRA inhibits estrogen-stimulated proliferation in uterine myometrium in vivo and in smooth muscle and fibroid tumor cells in vitro (Boettger-Tong and Stancel, 1995; Boettger-Tong et al., 1997). The results from our study demonstrated that both the primary tumors and the continuous cell lines derived from Eker rat leiomyomas expressed similar RXR and RAR profiles, suggesting that the ELT cell lines retain important signaling pathways seen in smooth muscle tumors in vivo.

In this study, we observed a reduction in the percentage of macroscopic tumors in treated animals compared with untreated control rats. This suggests that LGD1069 was effective in either slowing the progression of small microscopic leiomyomas to larger gross tumors or, alternatively, in enhancing the regression of large tumors. Our analytical methods do not allow us to distinguish between these effects, or to follow the progress of individual tumors over time. However, because the size of a tumor is a major factor in the severity of the symptoms (Buttram and Reiter, 1981), our results suggest that treatment with LGD1069 could reduce symptoms and possibly delay or avoid surgery.

In addition, our results indicate that the reduction in size of the tumors observed in LGD1069-treated animals was due to an increase in apoptosis rather than a decrease in proliferation. Although it is clear that LGD1069 treatment was associated with an increase in apoptosis, it is not clear whether apoptosis was a direct effect of the compound. Because of the extended treatment interval, the observed increase in apoptosis at 16 months may have occurred as a secondary response to LGD1069 exposure, possibly through modulation of growth factor activity or differentiation. Importantly, no statistically significant change in the rate of proliferation was observed, although the number of proliferating cells was higher, on average, in treated versus untreated tissues. This suggests that the underlying mechanism responsible for this effect was an increase in apoptosis rather than an inhibition of proliferation. This contrasts with the antiproliferative effect of ATRA reported for normal smooth muscle and fibroid cells (Boettger-Tong et al., 1997) and one explanation may be differences in the biology of signaling via the RXRs and RARs. Our data are consistent with that observed in other model systems in which the antitumor effects of RXR ligands appear to be predominantly apoptotic (Boehm et al., 1995; Nagy et al., 1995).

Studies have shown that RAR ligands can demonstrate antiestrogenic activity (Gottardis et al., 1996a,b). Pretreatment with ATRA for 3 days inhibited estrogen stimulation of the stroma and myometrium of immature rat uteri (Boettger-Tong and Stancel, 1995). Additionally, previous reports have indicated that LGD1069 exhibited antiestrogenic activity in various tissues and tumors. Our results, however, do not indicate that LGD1069 was effectively antiestrogenic in the endometrium of intact female rats in this study. LGD1069 failed to inhibit the CEH present in a large percentage of treated and control rats. This background lesion is due at least in part to estrogen as demonstrated by studies in beagle dogs showing induction of these lesions with hormone treatment (Attia, 1989). Further evidence of the contribution of ovarian steroids to the development of this lesion is provided by studies demonstrating that CEH in aged Eker rats was inhibited by selective estrogen receptor modulators that have an antiestrogenic effect in this tissue (Fuchs-Young et al., in preparation). The presence of CEH in many of the treated animals suggests that LGD1069 did not inhibit the proliferative effect of ovarian steroids on the endometrium of aged rats. The significance of vacuolization of the vaginal epithelium is not clear at this time. This morphological change has also been noted in animals treated for 4 months with selec-
tive estrogen receptor modulators and may, therefore, be a tissue response to chemically induced changes in the hormonal milieu. (Fuchs-Young et al., in preparation). Studies are underway to clarify the basis for this observed morphological change.

In summary, these data offer evidence of a novel approach to the treatment of leiomyomas that may prevent or delay the need for surgery. Because antiestrogens such as tamoxifen are antiproliferative in fibroblasts but do not induce apoptosis, the utility of these compounds for treating leiomyomas is limited (Burroughs et al., 1997; Walker et al., 2000). However, combinatorial treatment of leiomyomas with antiestrogens and compounds such as LGD1069 could result in a synergistic reduction in tumor size and a prolonged reprise from severe symptoms. In fact, in the rat mammary carcinoma model, nonefficacious doses of LGD1069 and tamoxifen when combined cause a significant regression of existing mammary tumors (Bischoff et al., 1998). Furthermore, the lack of significant toxicity in humans of LGD1069 (Miller et al., 1997) suggests that LGD1069 may be a favorable alternative to conventional palliative therapies for this refractory tumor.

References
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