LANREOTIDE PROMOTES APOPTOSIS AND IS NOT RADIOPROTECTIVE IN GH3 CELLS

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ABSTRACT

Somatostatin analogs are a mainstay of medical therapy in patients with growth hormone (GH) producing human pituitary tumors, and it has been suggested that somatostatin analogs may be radioprotective. We utilized growth hormone secreting rat GH3 cells to investigate whether a somatostatin analog may limit the effects of radiation on proliferation and apoptosis in vitro and on tumor growth in vivo. Treatment with lanreotide alone at doses of either 100nM or 1000nM for 48hours reduced clonogenic survival by 5-10%. Radiation alone produced a dose-dependent survival curve with a SF2 of 48-55%, and lanreotide had no effect on this curve. The addition of lanreotide resulted in a 23% increase in the proportion of apoptotic sub-G1 cells following irradiation (P<0.01). In a mouse GH3 tumor xenograft model, lanreotide 10 mg/kg moderately inhibited the growth of GH3 tumors, with a 4X tumor growth delay time that ranged from 4.5 to 8.3 days. Fractionated local tumor radiation alone significantly inhibited tumor growth and produced a tumor growth delay of 35.1 ± 5.7 days for 250cGy fractions. The combination of lanreotide, either antecedent to or concurrent, with radiation of 250, 200 or 150 cGy/fraction for 5 days inhibited tumor growth and produced the tumor growth delay times that were similar to radiation alone (p>0.05). Pretreatment with lanreotide had the most significant radiosensitizing effect. These studies demonstrate that the somatostatin analog lanreotide is not radioprotective in GH3 cells, and further studies are necessary to determine the impact of lanreotide on apoptosis.
INTRODUCTION

Somatostatin is a peptide hormone that exerts its biologic activity, including inhibition of hormone secretion, by interacting with a family of G protein receptors. Administration of somatostatin analogs, such as lanreotide and octreotide, that interact primarily with the subtype 2 and 5 somatostatin receptors, reduces hormone production in neuroendocrine tumors, such as growth hormone (GH) in pituitary somatotroph adenomas (acromegaly) and serotonin in gastrointestinal carcinoid tumors (Lamberts et al. 1996; Arnold et al. 2002; Freda et al. 2005). Clinical studies have also demonstrated that somatostatin analogs can inhibit the growth of a variety of solid tumors, including malignancies of the breast (Weckbecker et al. 1992; Ingle et al. 1996), prostate (Vainas 2001; Koutsilieris et al. 2004), and lung (Bombardieri et al. 1995), though clinical efficacy has been variable (Raderer et al. 1999; Hejna et al. 2002). Somatostatin analogs are a mainstay of medical therapy for a variety of neuroendocrine tumors with regard to reduction in pathologic hormone secretion and inhibition of tumor growth.

Acromegaly is an uncommon disorder characterized by excess secretion of growth hormone (GH), resulting in excessive growth of bone and soft tissues, multi-system co-morbidities, and heightened risk of premature mortality (Ezzat et al. 1994; Ben-Shlomo et al. 2001; Katznelson 2005). Over 90% of cases of acromegaly are caused by adenomatous growth of pituitary somatotroph cells. The preferred treatment for acromegaly is surgical excision, however, adjuvant medical therapy, including somatostatin analogs, and radiation therapy are often necessary for this disease.

Several retrospective, non-controlled studies suggest that somatostatin analogs may be radioprotective in acromegaly (Landolt et al. 2000; Pollock et al. 2002). The anti-proliferative effects of somatostatin analogs on neuroendocrine cells, including human pituitary GH-secreting adenomas in vitro (Miller et al. 1995; Danila et al. 2001; Hubina et al. 2006), may underlie this
finding. This raises the issue of whether somatostatin analogs should be maintained during radiation therapy.

To investigate further the question of possible radioprotection by somatostatin analogs, we studied the effects of the somatostatin analog lanreotide on the proliferation and radiosensitivity of GH producing tumor cells both in vitro and in vivo.

**METHODS**

*Cell Culture*

The rat GH3 pituitary tumor cell line was purchased from ATCC. Although it would be ideal to use dispersed human pituitary GH-secreting tumor cells in culture for these studies, there are no human pituitary GH-secreting cell lines that can be maintained in a differentiated state in vitro for sufficient time for colony formation assessment (Danila et al. 2000; Danila, Haidar et al. 2001). GH3 cells were maintained in DMED/F-12 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in a 37°C humidified incubator with a mixture of 95% air and 5% CO₂. All experiments were performed on exponentially growing cells with a doubling time of approximately 30 hours.

*In vitro Clonogenic Assay*

The dose-response of GH3 cells to the treatment of both lanreotide and radiation was characterized using a clonogenic assay. GH3 cells were detached with 0.05% trypsin-EDTA solution, counted and plated in 60mm Petri dishes (BD Biosciences, San Jose, CA) at appropriate dilutions of 100-100,000 cells/dish in triplicate in fresh growth media. Lanreotide (supplied by IPSEN, Milford, MA, USA) was added to the plates at final concentrations of 0-1000 nM. Cells
were irradiated with 0-10Gy at room temperature using a $^{137}$Cs source with a dose rate of 300 cGy/min. Following exposure to lanreotide or gamma radiation, the media was removed, and dishes were washed twice with PBS solution and filled with fresh growth media. After incubation at 37°C for 21 days, cells were stained with 0.25% crystal violet. Colonies containing $\geq$50 cells were counted under a dissecting microscope and survival curves were generated. The plating efficiency (PE) was calculated as the percentage of cells plated that grew into colonies. The surviving fraction (SF) was defined as the fraction of cells surviving an intervention, i.e. number of colonies/(number of colonies plated x PE).

For the irradiation experiments, Lanreotide at final concentrations of 100nM or 1000nM (determined by experiments shown in Figure 1) was added at 48, 24 or 0 hours before radiation. Cells were irradiated with 0-10Gy in the presence of lanreotide at room temperature with a Cs-$^{137}$ gamma irradiator. Following radiation, cells with 24-hour or 0-hour pre-exposure to lanreotide were incubated in lanreotide-containing media for an additional 24 or 48 hours, respectively. After a total of 48-hour exposure, lanreotide-containing media was removed, and dishes were washed twice with PBS solution and then filled with fresh growth media. Dishes that were irradiated without lanreotide exposure were also washed twice with PBS and refilled with fresh media. Cells were incubated for 21 days for colony formation.

**Apoptosis and Cell Cycle Analysis**

GH3 cells were placed in 60mm Petri dishes (500,000 cells/dish) and grown overnight. Lanreotide at 100nM was added at 48h, 24h, or immediately (0h) before radiation. Cells were irradiated with 10Gy gamma radiation at room temperature. Cells were collected 48h, 72h, 96h, and 168h after irradiation, and washed with cold PBS plus 5mM EDTA. Cells were resuspended in cold PBS-EDTA solution and fixed with cold 100% ethanol. After incubation for 30 min at
room temperature, cells were pelleted and treated with 100µg/ml of RNase A in PBS-EDTA solution for 30 min at room temperature. Propidium iodide (PI) was added to a final concentration of 50 µg/ml. The DNA content was analyzed with a FACScan flow cytometer (BD Biosciences, San Jose, CA). The percentage of cells in the sub-G1 (apoptotic), G1, S, and G2/M phases was calculated. Control cells without any treatment showed a consistent cell cycle distribution within 168h (data not shown).

**Mouse xenograft tumor model and therapy**

Male nude mice, 8 weeks old and 20-25 grams in body weight, were purchased from Charles River Laboratories (Hollister, CA). Mice were tested and found to be negative for specific pathogens. The mice were normally bred and maintained under specific-pathogen-free conditions, and sterilized food and water were available ad libitum. Mice were injected subcutaneously in the right flank with 5x10^6 tumor cells in 100 µl of a Hank’s solution and Matrigel 1:1 mixture (BD Biosciences, San Jose, CA). One tumor per mouse was inoculated. When tumors reached an average size of 120 mm³ (80-200 mm³), mice were randomly assigned to the different treatment groups. Five to 8 mice were used in each group. Lanreotide was injected subcutaneously (s.c.) at doses specified in each experiment. For radiation, the unanesthetized tumor-bearing mice were placed in individual lead boxes with tumors protruding through a cut-out window at the rear of each box. The radiation was delivered using a Philips RT-250 200 kVp X-ray unit (12.5 mA; Half Value Layer, 1.0-mm Cu) at a dose rate of 138 cGy/min. Tumors were locally irradiated with a dose of 150 - 250 cGy per fraction daily for 5 consecutive days as specified in each experiment. The length and width of the tumors were measured with calipers before treatment by the same investigator, and three times a week thereafter until the tumor volume reached at least 4 times (4X) the pre-treatment volume. The tumor volume was calculated using the formula: tumor volume (mm³) = π/6 x length x width². The tumor volume quadrupling (4X) time was determined
by a best-fit regression analysis. The tumor growth delay (TGD) time (in days) is the difference between the tumor volume quadrupling time of treated tumors compared to that of untreated control tumors. Both the tumor volume quadrupling time and tumor growth delay time were calculated for each individual animal and then averaged for each group. In some experiments, a complete response (CR) of tumors was recorded if a tumor completely shrunk to the point that it was not palpable at the end of the experiment. Body weight was measured twice a week. The mouse experiments described herein were approved by the Stanford University Administrative Panel for Laboratory Animal Care.

**Statistical Analysis**

The significance of differences between mean values obtained for the various study endpoints was calculated using an unpaired Student's *t* test.

**RESULTS**

**In vitro experiments**

**Dose responses of GH3 cells to lanreotide and gamma radiation**

As shown in Figure 1A, treatment with lanreotide resulted in a dose-dependent decrease in GH3 cell colony forming units. Lanreotide at doses of 1, 10, 100, and 1000nM resulted in cell survival rates of 75%, 56%, 39% and 27%, respectively. The IC50 (50% inhibition of cell growth) was 57nM. The radiation survival curves are shown in Figure 1B. GH3 cells had a typical radiation dose-response survival curve with an initial shoulder at doses below 5Gy and a straight line at high dose. The surviving fraction at 2Gy (SF2, a dose commonly used in daily fractionated radiotherapy) was 40%. Effect of lanreotide on radiation response of GH3 cells
GH3 cells were plated in tissue culture dishes overnight. The radiation survival curves are shown in Figure 2. Treatment with lanreotide alone at doses of either 100nM or 1000nM for 48 hours without radiation reduced clonogenic survival compared with untreated controls by 5-10%. Radiation alone without lanreotide produced a dose-dependent survival curve with a SF2 of 48-55%. Treatment with lanreotide at a dose of 100nM for 48 hours either before (48h pre-lanreotide and 24h pre-lanreotide) or at the time of radiation (0h pre-lanreotide) produced survival curves that were slightly shifted downward and separated at doses of 7-10Gy from the survival curve produced by radiation alone without lanreotide (Figure 2A) indicating that the radiation response of GH3 cells was enhanced by lanreotide. The surviving fraction at 10Gy was 0.0006, 0.00022, 0.00040 and 0.00042, respectively, for radiation alone, 48h pre-, 24h pre- and 0h pre-exposure to lanreotide (Figure 2C). However, treatment with 1000nM lanreotide did not alter the shape and slopes of the radiation survival curves, indicating there was no radioprotection (or radiosensitization) effect under these experimental conditions (Figure 2B).

**Effect of lanreotide and radiation on apoptosis and cells cycle distribution**

The percentage of cells in sub-G1, G1, S and G2/M phases at 48 hours was 1.4 ± 0.2%, 73.2 ± 1.0%, 8.4 ± 1.0% and 16.9 ± 1.8%, respectively. Treatment with 100nM lanreotide alone resulted in the sub-G1, G1, S and G2/M phase distribution of 2.28 ± 0.3%, 73.8 ± 1.1%, 7.72 ± 0.8% and 16.2 ± 0.5%, respectively, indicating that the cell cycle profile was not significantly affected by treatment with lanreotide compared with the untreated control, except for a moderate increase in apoptotic sub-G1 cells from 1.4% to 2.28%. Treatment with 10Gy radiation resulted in a decrease in the proportion of cells in G1 phase from 73.2% to 51.5% at 48h. Meanwhile, the G2/M phase cells increased from 16.9% before radiation to 35.7% at 48h after irradiation, and cells were arrested at G2/M phase for up to 168h without release. The subdiploid cell population, representing apoptotic cells, increased steadily following radiation from a baseline of 1.4% to a peak of approximately 12% at 168h (see Figure 3). Combined treatment of GH3 cells with
radiation and lanreotide produced a cell cycle profile that was similar to that seen in irradiated
cells without lanreotide, except for the increase in apoptotic sub-G1 proportion. As shown in
Figure 3, at 48h after irradiation, the apoptotic sub-G1 cells increased from 4.9% for radiation
alone to 8.6%, 9.3% and 13.4% for the combination of radiation with 48h, 24h and 0h pre-
exposure of lanreotide, respectively, representing an increase of 77% -173% compared with
radiation alone (P<0.01). At 168h after radiation, the sub-G1 cell fraction was 12% for radiation
alone and 20-22% for radiation plus lanreotide, representing an increase of 67% to 83% (P<0.01).

In vivo experiments

Dose responses of GH3 tumors to lanreotide

Groups of nude mice with established GH3 xenograft tumors were treated subcutaneously with
2.5, 5, 10, 20 or 50 mg/kg lanreotide daily for 5 days. Doses were based on prior studies utilizing
lanreotide administration in vivo (Prevost et al. 1994; Melen-Mucha et al. 2004). As shown in
Figure 4, there was a bell-shaped dose dependent effect of lanreotide on GH3 tumor growth, with
a narrow range of optimal doses. The maximum tumor growth inhibition (i.e. the longest tumor
growth delay (TGD) time of 13.1 ± 4.7 days) occurred with a daily lanreotide dose of 10 mg/kg.
When the daily lanreotide dose was either higher (i.e. 20 and 50 mg/kg) or lower (2.5 and 5
mg/kg) than 10 mg/kg, the effects of lanreotide on GH3 tumor growth were diminished.

In these studies, lanreotide at all doses tested did not cause significant decrease in body weight
compared to untreated control mice (data not shown). Also, there was no notable change in the
general appearance and daily activity of tumor-bearing mice treated with lanreotide.

Comparison of lanreotide dose regimen of once daily vs. twice daily

To determine the importance of lanreotide dose regimen on tumor growth, we compared tumor
size after single daily dose (qd) and two doses daily (bid). GH3 tumor-bearing nude mice were
injected s.c. with lanreotide at doses of 2.5, 5 or 10 mg/kg for 5 days either once daily or twice daily (8 hours interval). As shown in Figure 5, there were no statistically significant differences between groups treated either once daily or twice daily at the same dose (p=0.3-0.9). However, lanreotide at 10 mg/kg once daily produced the longest tumor growth delay time (4.9 ± 2.1 days) of all dose regimens studied (p<0.05). Notably, this was longer than that (1.1 ± 3.1 days) following 5 mg/kg twice daily. Analogously, a single daily dose of 5 mg/kg qd produced a longer tumor growth delay time than did 2.5 mg/kg bid. These data suggest that a single dose of lanreotide produced at least as much tumor growth inhibition than a fractionated dose regimen at the same total daily dose. Therefore, further studies utilized the single daily dosing regimen.

**Combination therapy of lanreotide and fractionated radiation**

To study the effect of lanreotide on tumor responses to radiation therapy, nude mice with established GH3 tumors were treated with 1) 10mg/kg lanreotide daily for 5 days; 2) local tumor radiation daily for 5 consecutive days at doses of 250, 200 or 150 cGy/fraction/day; 3) a combination of lanreotide and local tumor radiation as above; or 4) a s.c. injection of normal saline (0.005 ml/gram body weight) daily as an untreated control. In combination therapy, lanreotide was injected 20 minutes before radiation. Data are shown in Figure 6 (tumor growth curves). Lanreotide alone at a dose of 10 mg/kg moderately inhibited the growth of GH3 tumors, with a 4X tumor growth delay time that ranged from 4.5 to 8.3 days (p=0.3 ~ 0.06, compared to the relevant control groups). Fractionated local tumor radiation alone significantly inhibited tumor growth and produced tumor growth delay times of 35.1 ± 5.7 days for 250cGy fractions, 21.7 ± 5.5 days for 200cGy fractions, and 16.7 ± 1.7 days for 150cGy fractions, respectively. The combination of lanreotide with radiation of 250, 200 or 150 cGy/fraction for 5 days inhibited tumor growth and produced the tumor growth delay times that were similar to radiation alone (p>0.05). Also, the combined treatment of lanreotide and fractionated radiation did not cause any further decrease in animal body weight compared to fractionated radiation therapy alone.
Pre-administration of lanreotide in combination with radiation therapy

To study whether pre-administration of lanreotide could modulate radiation effects on tumor growth, nude mice with GH3 xenograft tumors were treated with 1) 10mg/kg lanreotide daily for 10 days; 2) 150cGy local tumor radiation daily for 5 consecutive days; and 3) 10mg/kg lanreotide for 5 days followed by combined administration of lanreotide and 150cGy radiation daily for 5 days. A group of tumor-bearing mice that was injected s.c. with normal saline daily for 10 days was also included as an untreated control. As shown in Figure 7, lanreotide at a dose of 10 mg/kg for 10 days moderately inhibited tumor growth (4X TGD, 8.3 ± 8.3 days, p=0.06 vs. control). Local tumor radiation of 150cGy inhibited tumor growth and gave a tumor growth delay time of 15.5.0 ± 8.8 days (p<0.05 vs. control and lanreotide alone). The combination therapy of pre-administration of lanreotide and radiation in this treatment regimen resulted in a tumor growth delay time of 15.1 ± 8.6 day, similar to that produced by radiation therapy alone (15.5 ± 8.8 days) (p>0.05). There were two mice with complete regression (CR) of tumors in both radiation alone and combination therapy groups, without tumor regrowth when the study was terminated after 60 days. Furthermore, lanreotide alone and in combination with radiation did not produce any obvious signs of systemic toxicity in terms of the loss of body weight, general appearance, skin reaction or activity level of the mice (data not shown).

DISCUSSION

Administration of Somatostatin analogs, such as lanreotide and octreotide, interact primarily with the subtype 2 and 5 somatostatin receptors and reduce hormone production in neuroendocrine tumors, such as G) in pituitary somatotroph adenomas that are associated with acromegaly (Lamberts, van der Lely et al. 1996; Arnold, Wied et al. 2002; Freda, Katznelson et al. 2005).
In an in vitro study of 18 human pituitary somatotroph adenomas, administration of somatostatin and its analogs inhibited proliferation in approximately 30% of tumors (Danila, Haidar et al. 2001). In this study, antiproliferative and anti-GH secretory activity did not always correlate, suggesting that the effects of somatostatin analogs on these biologic effects may be independent. Mechanisms underlying these anti-proliferative effects may include upregulation of the cyclin-dependent kinase inhibitor p27, inhibition of the MAP kinase pathway, mobilization of calcium, inhibition of PI3K/Akt survival signal and induction of the tumor suppressor Zac1 (Cheung et al. 1995; Danila, Haidar et al. 2001; Hubina, Nanzer et al. 2006; Theodoropoulou et al. 2006).

To study the effects of somatostatin analogs and radiation on tumor proliferation, it would be optimal to utilize human GH producing pituitary adenoma cells. However, dispersed human pituitary tumor cells are difficult to maintain for prolonged passages in culture (Danila, Zhang et al. 2000; Danila, Haidar et al. 2001). Studies have successfully utilized human fetal pituitary cells (Shimon et al. 1997), though this model is not representative of an adenoma cell line. Given the limitations of such models, we utilized rat GH3 cells because they express somatostatin receptors and are somatostatin responsive (Dasgupta et al. 1999). In addition, these cells grow well in culture and may be used in a xenograft tumor model. However, GH3 cells differ from human somatotroph cells in lacking functional GHRH receptors and expression of the somatostatin receptor subtypes (Zeytin et al. 1984; Garcia et al. 1994). Therefore, GH3 cells are not a precise model for investigating human pituitary disease, but these cells are relevant for such investigations and are highly useful when used in a model to demonstrate effects of somatostatin analogs on neuroendocrine tumor proliferation and growth.
In our study in vitro in rat GH3 cells, lanreotide had a dose-related anti-proliferative effect, with an IC50 of 57nM, in a colony formation assay. These results are similar to findings in another study using octreotide in GH3 cells using flow cytometric DNA analysis with propidium iodide staining (Cheung and Boyages 1995). In contrast, our findings differ from those of a previous study in GH3 cells in which octreotide did not show a dose response anti-proliferative curve, in that 1000 nM octreotide had reduced activity as compared with 100 nM octreotide (Pelicci et al. 1990). In the previous study, cells were exposed to octreotide for 24h, and the MTT assay was used. In contrast, we utilized the clonogenic assay, which may be more sensitive and reliable, and exposed the cells to lanreotide for a longer time; thus, potentially explaining our different findings. Of note, in our study, treatment with lanreotide did not change the cell cycle profile, in contrast to the production of partial G0/G1 cell cycle block reported by others (Cheung and Boyages 1995). Cheung and Boyages (Cheung and Boyages 1995) found the effect of octreotide on cell cycle arrest to be transient, lasting only 36-48 hours. In our study, cells were treated for 48 hours with lanreotide. It is possible that the difference between our results and those of Cheung and Boyages may reflect differences in the time of exposure to lanreotide, or differences in the action of lanreotide and octreotide.

Although lanreotide alone reduced proliferation of GH3 cells in a dose-responsive manner, lanreotide administration either before or during radiation had no effect on radiation survival in vitro. This is similar to a recent finding in another study (Rezacova et al. 2008). In fact, treatment with lanreotide at a dose of 100nM for 48 hours tended to shift the survival curves downward and increased the apoptotic cell population at the high doses of radiation. This suggests that lanreotide may synergistically enhance the radiation response of GH3 cells when combined with high dose radiation, such as that delivered clinically by stereotactic radiosurgery. Our data do not support a radioprotective effect of somatostatin analogs.
We utilized a mouse GH3 xenograft model to assess further both the antiproliferative effects of lanreotide and the potential effects of lanreotide on proliferation following radiation. Administration of lanreotide alone for 10 days resulted in moderate inhibition of tumor growth. This observation validated use of this model to assess the effects of somatostatin analogs on pituitary tumor cell proliferation. Lanreotide was well tolerated, as evidenced by the continued growth and weight of the animals. The anti-proliferative effect of lanreotide was observed irrespective of whether lanreotide was administered daily or as a split-daily dose, suggesting that anti-proliferative effects depend on the absolute daily dose, not the dose regimen. Of note, the anti-proliferative effects had a bell shaped dose effect, similar to that described previously with somatostatin analog administration in vitro with GH3 cells (Hubina, Nanzer et al. 2006). These data suggest that there may be a narrow range of doses that are optimal for maximizing the anti-proliferative effects of lanreotide.

The results presented here also demonstrate that co-administration of lanreotide with radiation at a variety of doses did not affect radiation response of GH3 tumors in vivo. In fact, several tumor-bearing mice in both the radiation and radiation plus lanreotide groups attained complete remission of tumors, which did not occur in the groups treated with lanreotide alone. These data suggest that somatostatin analogs, when administered at doses that produce a modest anti-proliferative effect in rat GH3 tumor model, are not radioprotective. Our data are also suggestive that administration of lanreotide prior to and during radiotherapy is more effective than administration of lanreotide during radiotherapy alone in reducing tumor volume, though we did not test this finding in a single experiment. Nevertheless, this finding is suggestive that administration of lanreotide prior to radiotherapy, which is reflective of clinical practice in humans, is highly effective at adding to radiation effects in reducing tumor volume. Further
studies of different somatostatin analog dosing regimens are needed to optimize the dosing of lanreotide when given in combination with radiation therapy. These studies are necessary to further confirm our initial findings that lanreotide does not radioprotect pituitary tumors in vivo. In addition, our studies may suggest paradigms for investigation of radiosensitizing effects of somatostatin analogs in pituitary and other tumor models.

In our study, lanreotide enhanced radiation-induced apoptosis in GH3 pituitary tumor cells. The apoptotic subdiploid cell population increased steadily following exposure to 10Gy radiation and 100nM lanreotide, indicating a super-additive effect on apoptosis with combined use of lanreotide and radiation. The mechanism of action by which lanreotide enhances radiation-induced apoptosis has not been elucidated. Previous studies have demonstrated that somatostatin analogs can up-regulate pro-apoptotic genes p53 (Sharma et al. 1996), death receptors (Guillermet et al. 2003), and BAX (Sharma et al. 1998), and down-regulate anti-apoptotic bcl-2 (Guillermet, Saint-Laurent et al. 2003).

p53 is the most commonly altered gene in human cancer. In response to DNA damage, p53 activates genes leading to apoptosis and cell cycle arrest. Even though lanreotide did not affect the cell cycle distribution, and blocks cells at G1 phase in our study, one cannot exclude a role for p53 in lanreotide-induced apoptosis in GH3 cells. In a previous report (28), Sharma et al showed that the somatostatin analog, octreotide, induces wild-type p53-associated apoptosis without G1 cell cycle arrest in CHO cells. The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a powerful apoptosis inducer. TRAIL induces apoptosis through activating its pro-apoptotic death receptor and effector caspase 3 (Ashkenazi et al. 1998; Luciani et al. 2005), independent of mitochondria (i.e. extrinsic pathway) (Scaffidi et al. 1998). Radiation induces
apoptosis mostly via the mitochondrial signal pathway (i.e. intrinsic pathway), an effect which can be blocked by over-expression of bcl-2. Since radiation and somatostatin analogs induce apoptosis in tumor cells through different signaling pathways, combination of the two modalities may lead to synergistic effect on apoptotic cell death. Furthermore, it has reported that somatostatin analogs inhibit the expression and secretion of the growth factors, such as IGF-1, VEGF and bFGF, in tumor cells (Baldysiak-Figiel et al. 2005; Kanashiro et al. 2005). Since ionizing radiation activates and/ or induces the over-expression of VEGF, bFGF and other growth factors in tumor cells (Kim et al. 2006), combined use of lanreotide and ionizing radiation could inhibit the pro-survival autocrine and paracrine effects of these radiation-induced growth factors in targeted tumor cells.

Our experimental paradigm may serve as a model to assess the radioprotective qualities of somatostatin analogs in acromegaly. Radiation therapy is often utilized as adjuvant therapy for persistent, active disease following incomplete surgery (Castinetti et al. 2005; Jenkins et al. 2006). Because patients are often symptomatic at the time of radiation therapy, somatostatin analogs are often administered in conjunction with radiation. However, there has been concern that the anti-proliferative effects of somatostatin analogs may protect the tumor cells from the tumoricidal effects of radiation. In a retrospective study, Landolt et al (Landolt, Haller et al. 2000) assessed the effects of gamma knife stereotactic radiosurgery (SRS) in 31 subjects with acromegaly, nine of whom received octreotide at the time of radiation. Following SRS, a smaller fraction of the patients treated with octreotide at the time of SRS achieved biochemical remission. The non-significant trend for higher GH and IGF-1 levels in subjects who required octreotide, suggests that the difference in response to SRS may reflect more aggressive disease in the subjects receiving octreotide. In contrast, the use of octreotide at the time of gamma knife SRS did not affect biochemical outcomes in 82 subjects with acromegaly in another study (Castinetti,
Taieb et al. 2005). Nevertheless, the question as to whether somatostatin analogs should be withheld at the time of radiation therapy remains controversial. Our findings in an animal model suggest that somatostatin analogs may in fact be radiosensitizing, a finding that clearly deserves more study.

In summary, using a mouse xenograft model, we have demonstrated that the somatostatin analog lanreotide does not protect pituitary tumor cells from the effects of ionizing radiation, but does promote radiation induced apoptosis in rat GH3 cells. Further studies, both in vitro and in vivo, are needed to determine the potential clinical relevance of these findings to the management of patients with neuroendocrine tumors, such as acromegaly, and whether somatostatin analogs should be administered concurrently with radiation therapy. These preliminary findings also suggest that lanreotide may enhance radiation-induced apoptosis and warrant further study. In addition, further studies are necessary to elucidate the mechanisms underlying this potential synergistic action of lanreotide and radiation on apoptosis induction.

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REFERENCES


Cheung NW and Boyages SC 1995 Somatostatin-14 and its analog octreotide exert a cytostatic effect on GH3 rat pituitary tumor cell proliferation via a transient G0/G1 cell cycle block. Endocrinology 136(10) 4174-81.


LEGENDS

Figure 1. Dose response of GH3 cells to lanreotide (A) and gamma radiation (B). GH3 cells were plated in Petri dishes in triplicate and treated with various doses of lanreotide or radiation. Cells were incubated for 21 days for colony formation. Data are presented as surviving fraction against dose on a log-linear plot. Data points represent the mean ± S.D. of three samples per dose point.

Figure 2. Radiation survival curves for GH3 cells treated with radiation alone or in the presence of lanreotide at doses of 100nm (A) or 1000nM (B). Lanreotide (Lan) was given 48hr or 24hr prior to or immediately (0h) before radiation (0-10Gy). The total exposure time for lanreotide was 48 hours. For A and B, open square, Radiation (RT) alone; solid diamond, Lanreotide administrated 48h pre-RT; solid triangle, Lanreotide administrated 24h pre-RT; solid circle, Lanreotide administrated immediately before RT (0h). (C) Radiation survival of GH3 cells treated with 10Gy radiation with or without 100nM lanreotide. Data represent the mean ± S.D. from two experiments. * p<0.01 compared with 10Gy radiation alone.

Figure 3. Cell cycle redistribution of GH3 cells after irradiation in the absence or presence of lanreotide. Lanreotide at 100nM was added to the media either 48 hr, 24 hr or immediately (0 hr) before radiation and kept in the media until collection of samples. The cell cycle was analyzed using FACScan flow cytometer. (A) Cell cycle histogram from FASCan flow cytometer. (B) Percent of cells in apoptotic sub-G1 phase of the cell cycle after irradiation (0-168 hours).

Figure 4. Dose response of GH3 xenograft tumors to lanreotide. GH3 tumor-bearing mice were injected with 2.5, 5, 10, 20 or 50 mg/kg lanreotide daily for 5 days. The 4X tumor
growth delay (TGD) times were calculated for each tumor and averaged for each dose point. Data are presented as TGD in days as a function of dose. Data points represent the mean ± standard deviation of two experiments.

Figure 5. Tumor growth curves of mice with GH3 tumors treated with lanreotide at doses of 2.5, 5, or 10 mg/kg once daily (qd) or twice daily (bid). Data are presented as the average tumor volume of each group (mean ± SD) versus time from start of treatment.

Figure 6. Tumor growth curves of GH3 tumor mice after combined treatment with lanreotide and fractionated local tumor radiation. Lanreotide (Lan) was injected subcutaneously (s.c.) at 10 mg/kg daily for 5 days. Radiation was delivered locally to the tumor at a daily dose of 250 (top), 200 (middle), or 150 (bottom) cGy for 5 days. There were four groups in each study: 1) untreated control (open square); 2) lanreotide alone (solid circle); 3) radiation alone (solid diamond); and 4) combination of lanreotide and radiation (solid triangle). Six to 8 animals were used in each group. Data are presented as the relative tumor volume: this consists of the ratio of tumor volume of each group (mean ± SD) compared to baseline tumor volume.

Figure 7. Tumor growth curves of mice with GH3 tumor treated with lanreotide and fractionated radiation. Lanreotide (Lan) was injected at a dose of 10 mg/kg daily for 10 days. Radiation was delivered locally to the tumor at a daily dose of 150cGy for 5 days. There were four groups: 1) untreated control (open square); 2) lanreotide alone (solid circle); 3) radiation alone (solid diamond); and 4) combination of lanreotide and radiation (solid triangle). Six animals were used in each group. The treatment paradigm is shown on top. Data are presented on the bottom as the relative tumor volume: this consists of the ratio of tumor volume of each group (mean ± SD) compared to baseline tumor volume.
Figure 3

A

Time after radiation exposure

0h 48h 72h 96h 168h

Control

Lanreotide

Radiation

48h pre-Lan + Radiation

24h pre-Lan + Radiation

0h pre-Lan + Radiation

B

Apoptosis (% total)

25
20
15
10
5
0

0 24 48 72 96 120 144 168

Hours after radiation

- Control
- Lanreotide
- Radiation
- 48h pre-lan +RT
- 24h pre-lan +RT
- 0h pre-lan +RT
Figure 5

![Graph showing relative tumor volume over days from start of treatment for different dosage regimens.]

- **Control**
- 2.5mg/kg qd
- 2.5mg/kg bid
- 5mg/kg qd
- 5mg/kg bid
- 10mg/kg qd
- 10mg/kg bid