Gabapentin-Induced Mitogenic Activity in Rat Pancreatic Acinar Cells

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Gabapentin induces pancreatic acinar cell tumors in rats through unknown, yet apparently nongenotoxic mechanisms. The primary objective of this study was to determine whether gabapentin acts as a tumor promoter by stimulating acinar cell proliferation in rat pancreas. To this end, indices of pancreatic growth, including increased pancreatic weight, stimulation of acinar cell proliferation, and/or enhanced expression of immediate-early oncogenes were monitored in rats given gabapentin in the diet at 2 g/kg/day for up to 12 months. Rats fed raw soy flour (RSF), a known inducer of pancreatic acinar cell tumors through cholecystokinin-mediated mitogenic stimulation, were used throughout as positive controls. In addition, recent data suggests that gabapentin binds to the α5 subunit of a voltage-gated, L-type calcium channel. Because signaling pathways for proliferative processes in pancreatic acinar cells involve intracellular calcium mobilization, the effects of gabapentin on intracellular calcium mobilization ([Ca2+]i) and 3H-thymidine incorporation were investigated in pancreatic acinar cells isolated from normal rat pancreas and in the AR42J rat pancreatic tumor cell line. As indicated by BrdU labeling indices, acinar cell proliferation increased 3-fold by Day 3 of RSF treatment and remained slightly greater than controls throughout the experiment. Pancreatic weights of RSF-fed rats were 32 to 56% greater than controls throughout the experiment. In contrast, gabapentin had no effect on pancreatic weight or acinar cell labeling index, and therefore had no apparent effect on pancreatic growth. In isolated pancreatic acinar cells, however, gabapentin induced mobilization of intracellular calcium and caused a slight increase in 3H-thymidine incorporation. The data suggest that gabapentin may possess low level mitogenic activity, which is not easily detectable in in vivo assays.

Key Words: acinar cell tumors; calcium mobilization; cholecystokinin (CCK).

The anticonvulsant gabapentin induces pancreatic acinar cell tumors in male rats (Sigler et al., 1995), but has no activity in bacterial or mammalian mutagenicity assays, in vitro chromosomal aberration assays, or in vivo micronucleus assays, and therefore it appears to act via nongenotoxic mechanisms. The gastrointestinal hormone cholecystokinin (CCK) is a potent trophic factor for normal pancreas and may also act as a promoter in pancreatic tumorigenesis (Douglas et al., 1989; Howatson and Carter, 1985). Stimulation of endogenous CCK levels by xenobiotics, such as the sulfonyl urea compound A8947, has been shown to result in pancreatic hypertrophy and hyperplasia (Obourn et al., 1997). Further, in an azaserine-induced rat pancreatic tumor model, enhanced expression of CCK-A receptors, as well as expression of CCK-B receptors not normally expressed in rat pancreas, have been demonstrated in preneoplastic and neoplastic pancreatic foci (Bell et al., 1992; Zhou et al., 1992). Hypothetically, these chemically induced changes in pancreatic acinar cell receptor populations may increase sensitivity to endogenous trophic hormones such as CCK or gastrin, leading to enhanced cellular proliferation and tumor promotion. In preliminary experiments, however, we were unable to discern any effects of chronic gabapentin treatment on plasma CCK levels or on expression of pancreatic CCK-receptor subpopulations (de la Iglesia et al., 1997).

The objective of this study was to evaluate the mitogenic potential of gabapentin toward pancreatic acinar cells and test the hypothesis that the drug may behave as a tumor promoter through increased cell proliferation. To this end, indices of pancreatic growth, including increased pancreatic weight, acinar and ductal cell proliferation, and enhanced expression of immediate-early oncogenes in pancreas were assessed in rats given gabapentin in the diet at levels that induce pancreatic neoplastic growth. In addition, autoradiographic data have show that the rat pancreas accumulates gabapentin transiently after single oral doses (Vollmer et al., 1986), although radioligand binding assays in normal pancreas did not reveal specific gabapentin binding sites (Suman-Chauhan et al., 1993; Gee et al., 1996). Recent data suggest that gabapentin binds to the α5 subunit of an L-type calcium channel (Gee et al., 1996). Because intracellular calcium ([Ca2+]i) plays an integral role in signaling pathways for proliferative as well as secretory processes in pancreatic acinar cells (Duan and Williams, 1994; Tsumoda and Owyang, 1995), we also examined the effect of gabapentin on [Ca2+]i mobilization and cell proliferation in isolated normal pancreatic acinar cells and in AR42J rat pancreatic acinar cells in vitro. These latter data suggest that gabapentin-induced pancreatic tumorigenesis in rats may yet...
be related to weak promotional effects manifested through enhanced pancreatic acinar cell proliferation.

MATERIALS AND METHODS

In Vivo Studies

Experimental animals. Three hundred and fifty-four male, random-bred, albino Wistar [Cr:W(BR)] were assigned randomly to groups and treated as indicated below until used for experimental analyses. Rats were housed individually in stainless steel cages and maintained in climate controlled rooms with 12-h light and dark cycles. Briefly, a group of 118 rats was fed gabapentin in the diet at 2000 mg/kg/day. One hundred and eighteen positive control rats were fed diet, ad libitum, containing raw soy flour (RSF) and 118 negative-control rats received Purina Certified Chow® (5002) until the time of termination, typically between 8 A.M. and 10 A.M. Subgroups of rats from each group were removed periodically for various assays as described below and in the tables and figure legends.

Pancreatic cell proliferation studies in vivo. Pancreatic acinar and duct cell proliferation was measured in control, RSF-fed, and gabapentin-treated rats after 3, 7, 14, 31, 91, and 274 days of treatment. Cell proliferation rates were determined as labeling indices by measuring the percentage of cell nuclei incorporating the thymidine analog, bromodeoxyuridine (BrdU) during S-phase of the cell cycle. To label cells entering S-phase, osmotic pumps (Alza, Model 2ML1) containing 50 mg/ml BrdU in 0.5 N NaCO₃ were implanted subcutaneously 4 days prior to scheduled sacrifice. Upon necropsy, pancreata were weighed and examined macroscopically. Representative samples of morphologically normal-appearing pancreatic tissue were collected and processed for histopathological assessment and for morphometric determination of BrdU labeling indices. Measurements of acinar and ductal cell densities were also obtained by morphometry.

In Vitro Studies

Immediate-early oncogene analyses. Individual samples of pancreas or pancreatic nodules were collected on Day 3 and during Weeks 4, 39, 52, and 91 and stored at –20°C until use. [32P]-labeled RNA probes were synthesized from 15 min at 14,000 rpm of a panbroth lysate from AR42J rat pancreatic tumor cells obtained by morphometry.

Pancreatic acinar cell cultures. AR42J rat pancreatic tumor cells obtained from American Type Tissue Culture were grown in standard tissue culture flasks in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum. Primary cultures of normal acinar cells were prepared from rat pancreas according to collagenase digestion methods described by Lu and Logsdon (1992). Normal acinar cells were plated overnight in collagen-coated dishes in Waymouth’s medium containing 10% fetal calf serum. Cultures were maintained in a humidified atmosphere of 95% oxygen/5% CO₂ at 37°C.

In vitro calcium mobilization. Effects of gabapentin on calcium mobilization were evaluated by laser cytometry (ACAS, Meridian Instruments, Okemos, MI) in normal pancreatic acinar cells and in AR42J pancreatic tumor cells. Calcium flux was measured by using the acetoxymethyl ester form of the fluorescent calcium chelator Fluo-3 at excitation and emission wavelengths of 488 and 515 nm, respectively. Cells were plated in 24-well tissue culture dishes at a density of 2 × 10⁴ cells/well. Cells were pretreated with the fluorescent Ca²⁺ indicator at 5 mM for 30 min, rinsed once, and covered with phenol red-free DMEM and treated with CCK-8 and/or gabapentin, as described in the figure legends.

Cholecystokinin receptor competitive binding assays. To test the hypothesis that gabapentin’s effects on intracellular calcium mobilization could be mediated by interactions at CCK receptors, ligand competition binding assays were conducted on normal rat pancreatic membranes as described above using 35 pM ¹²⁵I-BH-labeled CCK-8 in the presence of increasing concentrations of unlabeled CCK-8 or gabapentin ranging from 10⁻¹⁶ to 10⁻⁷M.

Cell proliferation in vitro. Effects of gabapentin on proliferation of normal pancreatic acinar cells and AR42J rat pancreatic tumor cells was assessed by 3H-thymidine incorporation. Cells were plated at 60,000/cm² in 12-well tissue culture dishes and incubated overnight. Medium was replaced with treatment solutions containing 1 µCi ³H-thymidine and increasing concentrations of gabapentin as indicated in the figure legends. Cells were incubated for 18 h, after which trichloroacetic acid-precipitable radioactivity was quantitated by scintillation spectroscopy.

Statistical analyses. Treatment groups were compared using a one-way analysis of variance (ANOVA) with Student-Newman-Keuls post-hoc test, where appropriate. A p-value < 0.05 was selected prior to the study, for determining statistical significance.

RESULTS

In Vivo Experiments

Pancreas weight. Rats fed normal diet exhibited time-dependent increases in pancreas weight consistent with normal organ growth in young rats. Pancreas weights in gabapentin-treated rats were comparable to controls throughout the study. In contrast, pancreatic weights in RSF-fed rats were 46, 32, 35, 56, 56, and 43% greater than controls on Days 3, 7, 14, 31, 91, and 274, respectively (Fig. 1).

Pancreatic acinar cell proliferation in vivo. Acinar cell labeling indices for controls and gabapentin-treated rats were essentially constant over the first 14 days of the study and ranged from approximately 8 to 15% (Fig. 2). Thereafter, acinar cell labeling indices in these rats decreased with time to less than 1% by Day 274. Relative to controls, acinar cell labeling index in RSF-fed rats increased 3-fold to approximately 33% by Day 3 of treatment and decreased toward control levels by Day 7. In general, acinar cell labeling indices in morphologically normal pancreatic tissue from RSF-fed rats were typically greater than controls, particularly on Days 31, 91, and 274, although the differences were not statistically significant. Increased pancreatic weight in RSF-fed rats was accompanied by pancreatic acinar cell hypertrophy, which was
observed microscopically and reflected by decreases in acinar cell density (Table 1). Duct cell labeling indices appeared to show a similar time-dependent decrease, but the data were highly variable and there were no differences between treatment groups (Table 1).

**Emergence of pancreatic nodules.** Pancreatic nodules were generally detectable once they achieved a size of 2 mm. Nodules in the 2- to 3-mm size range were observed in one animal each in gabapentin- and RSF-fed groups at Day 31. At Day 91, additional, widely scattered nodules in this size range were observed, but in the RSF-fed group only. By Day 274, multiple 2- to 3-mm nodules were observed in gabapentin-treated as well as RSF-fed groups. Rats in the RSF-fed group also had nodules in the 3- to 8-mm range. Pancreatic nodules in untreated controls were rare, were not detected until Day 274, and were in the 2- to 3-mm size range. Nodules in the 2- to 5-mm range were considered foci of acinar cell hyperplasia based on histological characteristics. Typically, these nodules were characterized by well-differentiated exocrine cells forming normal acini and ducts. Nodules were usually devoid of islet cells. Pancreatic nodules in the 5- to 8-mm range were sharply demarcated from surrounding tissue, had well-differentiated acinar cells displaying a mild degree of nuclear pleomorphism, and exhibited crowding with variable cell size.

**Immediate-early oncogene expression.** Expression of immediate-early oncogenes c-myc, c-fos, or c-jun in grossly normal pancreas tissue collected from RSF-fed or gabapentin-treated rats after 3, 31, or 274 days of treatment was comparable to controls and there were no apparent time-dependent trends (data not shown).

Immediate-early oncogene expression was also evaluated in pancreatic nodules. Nodules from untreated controls and from gabapentin-treated rats were approximately 2 to 3 mm in size while nodules from RSF-fed rats ranged from 5 to 8 mm. Compared to normal tissue, oncogene expression was increased 2- to 4-fold in some pancreatic acinar cell nodules, whether harvested from control, RSF-fed, or gabapentin-treated rats (Figs. 3A–3C). Relative to normal tissue from untreated rats and relative to morphologically normal tissue neighboring the nodules, expression of c-myc, c-fos, and c-jun increased in these nodules from control and gabapentin-treated rats, while only c-myc and c-jun increased in nodular tissue from RSF-fed rats. Levels of c-fos in tissue from RSF-fed rats were comparable to morphologically normal pancreatic acinar tissue.

Increased oncogene expression was not observed in all pancreatic lesions, however. C-myc and c-jun increased in nodules harvested from RSF-fed rats after 52 weeks of treatment, but not in nodules obtained after 94 weeks. Conversely, c-myc and c-jun increased relative to morphologically normal tissue in pancreatic nodules taken from gabapentin-treated rats after 94 weeks, but not in nodules obtained after 52 weeks. In contrast to nodules from RSF-fed rats in which expression of c-fos was not increased, c-fos increased in nodules from gabapentin-treated rats after both 52 and 94 weeks of treatment. Relative to normal tissue, expression of all 3 immediate-early oncogenes increased in nodules obtained from control rats after 94 weeks.

**In Vitro Experiments**

**Effects of gabapentin on calcium mobilization.** As indicated by a sharp increase in Fluo-3 fluorescence, CCK-8 at $10^{-8}$ M induced a rapid mobilization of calcium in AR42J cells,
followed by an equally rapid return toward baseline levels (Fig. 4A). Gabapentin alone had no effect at 0.5 \times 10^{-7} \text{M}, but inhibited CCK-stimulated calcium transients. Subsequent addition of the calcium ionophore, ionomycin, at 2 \mu M confirmed the ability of gabapentin-treated cells to generate a calcium response (Fig. 4B). Sequential treatment of AR42J cells with CCK-8 and gabapentin demonstrated a rapid inhibition of CCK-8 induced calcium flux (Fig. 4C).

Similar to the response observed in the AR42J cells, normal pancreatic acinar cells rapidly mobilized calcium in response to stimulation with 10^{-8} \text{M} CCK-8 (Fig. 5A), but the response in normal acinar cells appeared more robust than in the neoplastic cells. Like CCK-8, but in contrast to its effects on calcium mobilization in AR42J cells, gabapentin alone at 10^{-8} \text{M} stimulated rapid and relatively long-lasting increases in intracellular calcium and appeared not to block CCK-8-stimulated calcium mobilization in normal acinar cells (Fig. 5B). Continued responsiveness to CCK-8 stimulation could be demonstrated after exposure to gabapentin at 10^{-7} \text{M} (Fig. 5C).

**CCK receptor competition assays.** To determine whether gabapentin’s inhibitory effects on AR42J cells and stimulatory effects in normal acinar cells were related to CCK receptor activity, competitive receptor binding studies were conducted.
in membranes isolated from normal rat pancreas using 35 pM $^{125}$I-BH-CCK-8S in the presence of increasing concentrations of unlabeled gabapentin (Fig. 6). Binding of CCK-8S was unaffected by gabapentin at concentrations as great as 1 mM, indicating that gabapentin does not bind with affinity to the CCK receptor, and thus is likely to act at a different site to modulate calcium mobilization.

Pancreatic acinar and AR42J tumor cell proliferation in vitro. Dose-related increases in $^3$H-thymidine incorporation into normal pancreatic acinar cells ranging from 240% to 320% were observed after 18-h incubations at gabapentin concentrations from 1 to 100 μg/ml, with statistically significant differences from control at 1 and 10 μg/ml (Fig. 7). Mean values for $^3$H-thymidine incorporation also appeared increased at 100 μg/ml, but there was great variability in the data and differences from control were not statistically significant. Thymidine incorporation into normal acinar cells decreased below control levels at gabapentin concentrations of 1- and 10-mg/ml, most likely due to cytotoxicity at these extremely high doses. In contrast, gabapentin had no effects on proliferation of AR42J cell proliferation, in which $^3$H-thymidine incorporation was comparable to controls at doses ranging from 0.01- to 0.1-mg/ml. Similar to control acinar cells, thymidine incorporation in AR42J cells decreased below control levels at gabapentin concentrations of 1 mg/ml and above.

**DISCUSSION**

In two-year carcinogenicity studies, gabapentin at 2000 mg/kg/day caused a statistically significant increase in acinar cell tumors in male Wistar rats, but not in female rats or B6C3F1 mice (Sigler et al., 1995). Based on cytological features, a number of these neoplasia were categorized as acinar cell carcinomas, but the tumors did not metastasize. Onset and latency for gabapentin-induced tumors were the same as those in concurrent controls, and the tumors had no effect on survival or morbidity. Common exocrine pancreatic tumors in Wistar rats and in humans differ significantly based on the pathobiological characteristics. Rats develop acinar cell tumors (Rao, 1987), whereas humans manifest the highly malignant and
more aggressive duct cell carcinoma (Bockman, 1981; Maru-
chi et al., 1979). Based on the negative genotoxicity profile of
gabapentin, pancreatic carcinogenesis in gabapentin-treated
rats was considered to be an epigenetic phenomenon, and the
weight of evidence suggests the potential for human risk to be
very low.

The tumor biology of gabapentin-induced pancreatic acinar
tumors appears similar to neoplasia occurring in rats sponta-
neously and in those given trypsin inhibitors or soya flour in
the diet (Gumbmann et al., 1989; McGuinness et al., 1980).
Cholecystokinin is a trophic factor for pancreas and has been
implicated as a promoter in pancreatic carcinogenesis in several
rodent models (Douglas et al., 1989; Howatson and Carter,
1985). Feeding soybean trypsin inhibitor contained in raw soy
flour disrupts the trypsin-dependent negative feedback mech-
anism normally controlling CCK release and results in aug-
mented CCK levels and pancreatic growth (Goke et al., 1986).
Prolonged elevations in CCK caused by chronic trypsin inhi-
bition in rats fed raw soya flour induces pancreatic acinar cell
hyperplasia, adenomas, and carcinomas (Herrington et al.,
1994). CCK has also been shown to enhance the development
and shorten the induction time of preneoplastic acinar lesions
in azaserine-treated rats (Povoski et al., 1993). Further, en-
hanced expression of CCK-A receptors or novel emergence of
CCK-B/gastrin receptors could render neoplastic tissue more
sensitive to stimulation by endogenous CCK or abnormally
responsive to gastrin stimulation as described in the azaserine
model of pancreatic carcinogenesis (Bell et al., 1992; Zhou et
al., 1992). In separate, preliminary studies, we were unable to
discern changes in CCK concentrations or in CCK receptor
populations in pancreas of gabapentin-treated rats at

FIG. 6. CCK-receptor competition assays with gabapentin. To determine
whether gabapentin’s inhibition or stimulation of calcium mobilization in
AR42J cells and normal acinar cells, respectively, may be related to interac-
tions at the receptor level, receptor binding studies were conducted on normal
rat pancreatic membranes using 35 pM ^125-I-Bolton Hunter-labeled CCK-8S in
the presence of increasing concentrations of unlabeled gabapentin. Curves
represent means ± standard deviation, where n = 4.

FIG. 7. Effect of gabapentin on normal acinar and AR42J tumor cell
proliferation. Cell proliferation was evaluated using tritiated thymidine incor-
poration. Cells were plated at 60,000/cm² in 12-well tissue culture dishes and
incubated overnight. Medium was replaced with treatment media containing 1
μCi ^3H-thymidine and increasing concentrations of gabapentin as indicated.
Cells were incubated for 18 h and trichloroacetic acid-precipitable radioactiv-
ity was quantitated by scintillation spectrometry. *p < 0.05 where n = 3,
Student-Newman-Keuls test.
Week 94, but not at Week 52, indicating that long-term treatment is needed and that the critical period of development lies between 1 and 1.5 years. Based on these cumulative data, gabapentin had no mitogenic effects demonstrable in vivo. Further, the data suggest that CCK does not play a significant role in gabapentin-induced pancreatic carcinogenesis.

Autoradiographic studies have shown that gabapentin accumulates transiently in pancreas following oral administration (Vollmer et al., 1986), but radioligand binding assays have not detected significant specific gabapentin binding sites in normal rat pancreas (Gee et al., 1996; Suman-Chauhan et al., 1993). Despite this absence of specific gabapentin binding sites in normal pancreatic tissue, it appears from our laser cytometric data that gabapentin alters normal pancreatic acinar cell calcium homeostasis. Further, the data suggest fundamental differences in the profile of calcium responses between normal pancreatic acinar cells and AR42J pancreatic tumor cells. Specifically, gabapentin increases intracellular calcium levels in normal pancreatic acinar cells and has no apparent effect on CCK-stimulated intracellular calcium mobilization. In contrast, in the AR42J rat pancreatic-tumor cell line, gabapentin alone has no apparent effect on calcium levels, yet inhibits CCK-stimulated intracellular calcium mobilization.

Recent data indicates that gabapentin binds to the α,β subunit of a voltage-gated calcium channel, which is present in brain and skeletal muscle, but is not expressed in normal rat pancreas (Gee et al., 1996). The presence of L-type calcium channels has been demonstrated in the AR42J rat pancreatic tumor cell line, however (Christophe, 1994). It is conceivable that gabapentin might confer some growth advantage to acinar cells abnormally expressing L-type calcium channels through spontaneous mutation, but the presence of these binding sites in pancreatic acinar cell tumors from gabapentin-treated rats remains to be demonstrated. In the pancreas, calcium is a second messenger in the signaling cascade mediating acinar cell responses to trophic hormones, such as CCK (Louie, 1994). For example, the activated CCK receptor couples to G proteins and stimulates phospholipase C to catalyze the breakdown of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 stimulates the release of [Ca2+]i, while DAG activates protein kinase C in the plasma membrane. These latter events may act synergistically due to mutual molecular targets for Ca2+-calmodulin-dependent phosphorylation as well as phosphorylation by protein kinase C. Additional events in the acinar cell proliferative response include stimulation of tyrosine kinase, activation of mitogen-activated protein kinase activity, and expression of immediate-early response genes such as c-myc, c-fos, and c-jun (Lu and Logsdon, 1992). Although we have demonstrated that gabapentin does not interact with CCK receptors, its ability to increase intracellular calcium suggests that gabapentin may activate postreceptor downstream effectors and trigger proliferative signaling pathways. Using incorporation of 3H-thymidine as an indicator of S-phase activity and cell proliferation, the in vitro data support the notion that gabapentin may stimulate DNA synthesis in normal pancreatic acinar cells. Concentrations at which gabapentin stimulated 3H-thymidine incorporation in normal acinar cells are comparable to the plasma concentrations of approximately 110 µg/ml, associated with increased pancreatic acinar cell tumors in rats (Sigler et al., 1995). Acting through this mitogenic pathway, gabapentin may behave as a weak tumor promoter, as has been established for CCK (Douglas et al., 1989; Howatson and Carter, 1985).


