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Gene Expression of Gonadotropin-Releasing Hormone and Its Receptor in Rat Pancreatic Cancer Cell Lines

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The regression of experimental and clinical pancreatic cancers by treatment with gonadotropin-releasing hormone (GnRH) agonists or antagonists has been repeatedly reported and is usually presumed to result from the creation of a sex steroid deficiency. There are, however, indications that GnRH analogs can also suppress the growth of the tumor cells in vitro and that specific binding sites for GnRH are present on membranes of these cells. The regulatory role of GnRH in rat pancreatic adenocarcinoma was investigated by examining the gene for GnRH and GnRH receptor (GnRH-R) in two pancreatic tumor cell lines (AR42J and ARIP). Reverse transcriptase polymerase chain reaction and Southern blot analysis indicated both GnRH-mRNA and GnRH-R-mRNA transcripts in the two cell lines. This is the first report raising the possibility of an autocrine/paracrine role for GnRH in rodent malignant pancreas.

Key Words: Gene expression; gonadotropin-releasing hormone; gonadotropin-releasing hormone receptor; reverse transcriptase polymerase chain reaction; pancreatic tumor; mRNA.

Introduction

Inhibition of the growth of sex hormone–dependent tumors by analogs of gonadotropin-releasing hormone (GnRH) has been demonstrated experimentally and clinically *(1,2)*. The use of GnRH analogs for treatment of prostatic, ovarian, and breast cancers is based on suppression of pituitary-gonadal function and the consequent creation of a state of sex steroid deficiency. In addition to these indirect effects, GnRH agonists and antagonists exert direct effects on these tumors that most probably are mediated by specific GnRH receptors (GnRH-Rs) found on these cells *(3,4)*.

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Since 1984, it has been shown repeatedly that GnRH analogs also suppress the growth of experimental pancreatic cancers *(5,6)*. Exocrine pancreatic cancers could be sex steroid sensitive *(7)*, and the regression of experimental pancreatic cancers by treatment with GnRH agonists or antagonists could be explained, in part, by the deprivation of estrogen or androgen. Direct effects mediated by GnRH-Rs may also be involved since GnRH analogs inhibit the growth of Mia PaCa-2 pancreatic cancer cells in vitro. Hamster and human pancreatic tumors but not normal pancreata exhibit cell-membrane receptors for GnRH *(8)*. Therefore, we suggest that the presence of GnRH-Rs in pancreatic tumors could be involved in complex interactions that contribute to the growth or even the occurrence of cancer cells, and there might exist an autocrine/paracrine system based on GnRH in pancreatic cancer, as has been proposed for breast, prostate, and ovarian cancers *(9,10)*.

The possible role of GnRH in rat pancreatic tumors was investigated using reverse transcriptase polymerse chain reaction (RT-PCR) to examine gene expression of GnRH and GnRH-R in the AR42J and ARIP cell lines, both of which were derived from exocrine tumors in rat pancreas. To our knowledge, there still has been no report concerning the detection of GnRH-mRNA or GnRH-R-mRNA in rat pancreatic cancer cell lines, although we recently reported the presence of GnRH and its mRNA in normal rat pancreas *(11)*.

Results

Expression of GnRH mRNA in Rat Pancreatic Cancer Cells

The expression of GnRH mRNA in rat pancreatic tumor cell lines was examined using RT-PCR, with primers derived from rat hypothalamic GnRH cDNA *(12)*. As shown in Fig. 1A, primer F1 is located in the second exon corresponding to a specific region of the signal peptide and primer R1 is complementary to a segment of 3'-untranslated region that is encoded by exon 4. Amplification of cDNAs from AR42J and ARIP cells by primers F1 and R1 both produced 356-bp fragments. There was no band in lane 4 for PCR carried out in the absence of cDNA (Fig. 2). Amplification of the same cDNA samples with actin primers produced an abundant single product, confirming that there was no degradation in the RNA preparation (data not shown).

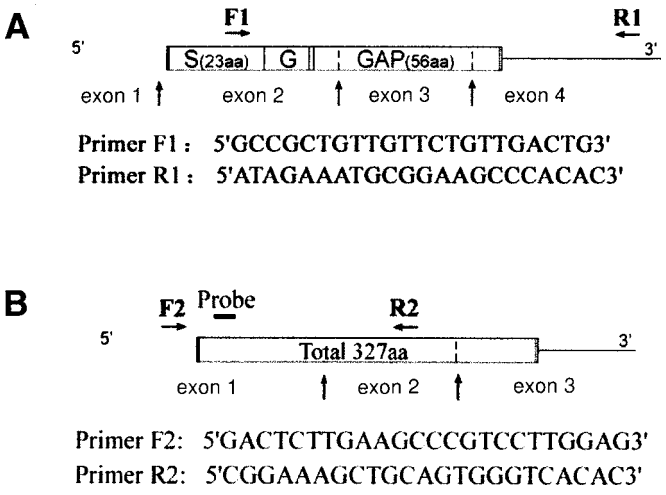

Fig. 1. Schematic structure of rat GnRH and GnRH-R cDNAs with positions and sequences of primers. Verticle arrows indicate the intron position, the box represents the coding region, and the lines represent the 5'- and 3'-untranslated regions. (**A**) Demonstration of rat GnRH mRNA. S, signal peptide (23aa); G, GnRH peptide (10aa); GAP, GnRH-associated peptide (56aa). (**B**) Demonstration of rat GnRH-R mRNA, encoding a total of 327 amino acids.

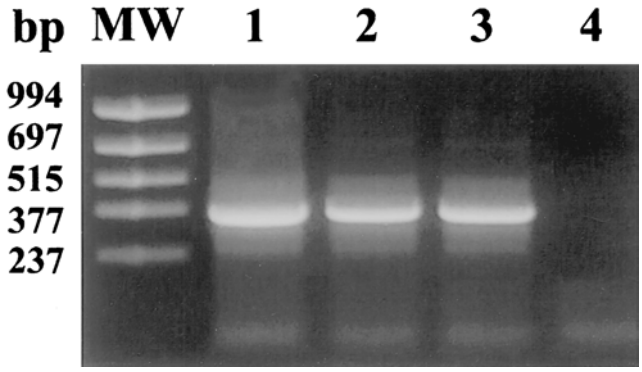

Fig. 2. Ethidium bromide–stained gel showing PCR products for GnRH. Total RNAs from rat hypothalamus (lane 1), AR42J cells (lane 2), and ARIP cells (lane 3) were reverse transcribed into cDNAs and subsequently amplified in PCRs with primers F1 and R1 for 35 cycles. All produced the predicted 356-bp bands. The negative control (lane 4) was amplified using the same conditions without the cDNA template.

Sequence analysis for the PCR product of ARIP purified from the agarose gel (data not shown) revealed that the purified cDNA was identical to published rat hypothalamic GnRH cDNA *(12)*.

Expression of GnRH-R mRNA in Rat Pancreatic Cancer Cells

cDNA produced from the total RNA isolated from rat pituitary and pancreatic cancer cell lines was amplified by PCR using oligonucleotides F2 located on exon 1 and R2 on exon 2 (Fig. 1B). The PCR products, 643 bp in size, were clearly detectable with ethidium bromide staining under ultraviolet light in all the samples but not in the negative control (Fig. 3).

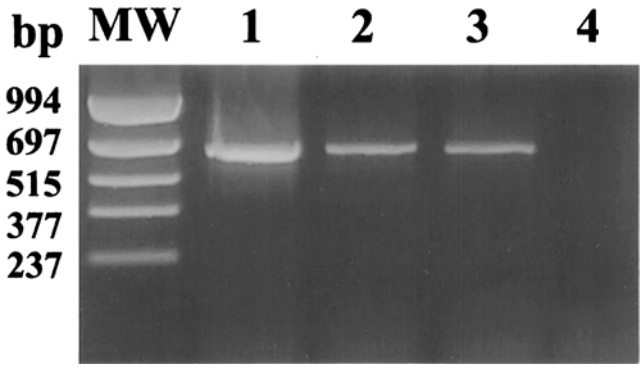

Fig. 3. Ethidium bromide–stained gel showing PCR products for GnRH-R. Total RNAs from rat pituitary (lane 1), AR42J cells (lane 2), and ARIP cells (lane 3) were reverse transcribed into cDNAs and subsequently amplified in PCRs with primers F2 and R2 for 35 cycles. All produced the predicted 643-bp bands. The negative control (lane 4) was amplified using the same conditions without the cDNA template.

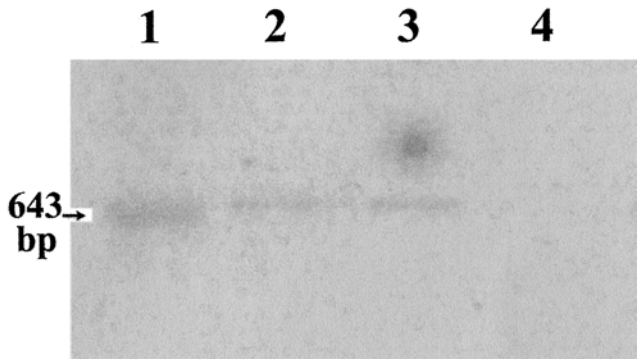

Fig. 4. Autoradiographs of GnRH-R PCR products after transfer to a nylon membrane and hybridization with ^{32}P-labeled internal sequence oligonucleotide. The 643-bp bands were detected in the rat pituitary gland sample (lane 1) as positive control and in both the AR42J (lane 2), and ARIP (lane 3) cell line samples, but not in the negative control (lane 4).

The products, on transfer to nylon membranes, hybridized to an internal oligonucleotide probe (Fig. 1B), thus confirming that the detected signals were for the GnRH-R (Fig. 4). The PCR product generated from total RNA of the ARIP cell line was further analyzed by sequencing (data not shown) and found to be identical to the published human pituitary GnRH-R sequence *(13)*.

Discussion

Our study has demonstrated, for the first time, the coexpression of mRNA for GnRH and GnRH-R in rat pancreatic cancer cells. RT-PCR combined with Southern blotting was used because hormone receptor genes are in general expressed in very low amounts, often below the detection limit of Northern hybridization. In addition, possible RNA

degradation during RNA preparation was controlled by systematic RT-PCR testing for the presence of the abundant β-actin transcript. This procedure has been successfully used to determine the molecular characteristics of GnRH and its receptor in different kinds of sex hormone–dependent tumors, such as breast carcinoma cell lines ZR75-1 and MCF-7, ovarian adenocarcinoma cell line SKOV3, prostate carcinoma cell lines PC-3 and LNCaP, and endometrial carcinoma cell lines RL95-2 and HHUA *(14,15)*. All these carcinoma cell lines germinate from secrete tissues that have similar tectology. They are known to have binding sites for GnRH and their proliferation is retarded by GnRH analogs *(3,4)*. The existence of an autocrine system based on the production of GnRH and the expression of its receptors has been proposed in many articles concerning different carcinoma cell lines and is being supported by new evidence. The antiproliferative effects of GnRH analogs on these cancer cells in vitro as well as the favorable response of some patients with recurrent cancer to GnRH analog treatment *(16)* might be reasonably explained by the interference with this autocrine system.

Early in 1989, Fekete et al. *(8)* reported the existence of GnRH-Rs in experimental *N*-nitrosobis-(2-oxopropyl)-amine-induced pancreatic cancers of hamsters and human pancreatic cancer obtained for autopsies. Although the functional role of the receptor in pancreatic carcinoma is still obscure, the effect of GnRH analogs has been reported to be linked to the enhanced ratio of programmed cell death (apoptosis) in the tumors *(17)*. Because of its short half-life, GnRH is undetectable or detected at very low level in the general circulatory system; thus, it is unlikely that endogenous hypothalamic GnRH ever reaches the necessary concentrations in peripheral blood to stimulate pancreatic tumor GnRH-Rs. Consideration of this fact led to the detection of GnRH-like molecules in human pancreatic tumors by immunocytochemistry *(18)*. The present study further established the authenticity of GnRH and its receptor in rat pancreatic cancer cell lines by demonstrating their mRNA using PCR. This demonstration was based on the generation of PCR products that are identical to GnRH cDNA from the hypothalamus or GnRH-R cDNA from the pituitary, well-established sites for GnRH and GnRH-R.

In conclusion, our data show that rat pancreatic carcinoma cell lines can express both GnRH and its receptor and that GnRH might act as an autocrine regulator of pancreatic carcinoma proliferation. We conjecture that a relatively high dose of GnRH analog might induce desensitization to GnRH or downregulation of the GnRH-R with a consequent decline in tumor growth, in an analogous manner to the action of GnRH analog on anterior pituitary and other sex hormone–dependent tumors. We believe further clarification of the mechanisms by which GnRH regulates the proliferation of pancreatic cancer cells should accelerate the development of efficacious therapeutic methods based on GnRH analogs.

Materials and Methods

Cell Lines

Two rat pancreatic carcinoma cell lines were obtained from American Type Culture Collection (ATCC). As indicated by ATCC comments, cell lines AR42J and ARIP were both derived from exocrine tumors in rat pancreas, of which AR42J can produce amylase and other exocrine enzymes, while ARIP can produce some exocrine enzymes at very low levels. Cells were grown at 37°C in Ham's F12K medium containing 20% (AR42J) or 10% (ARIP) fetal bovine serum in a 150-cm^2 flask in a humidified atmosphere of 5% carbon dioxide and 95% air.

RNA Preparation and RT-PCR

Total RNA was extracted using an RNAgents Total RNA Isolation System Kit from Promega. Approximately 50 μg of total RNA could be isolated from 5×10^6 cells. The integrity of RNA was determined by fractionation on agarose gel and staining with ethidium bromide.

Altogether 2 μg of total RNA from each sample was reverse transcribed into cDNA using the Reverse Transcription System Kit from Promega in 20 μL of reaction mixture containing oligo dT primer and AMV reverse transcriptase at 42°C for 30 min.

Primers specific for GnRH and GnRH-R (Fig. 1) were designed based on the published rat hypothalamic GnRH and pituitary GnRH-R cDNA sequences *(12,13)*. To exclude the effect of DNA contamination on the PCR result, the primers were designed to span putative exon-intron boundaries to allow specific amplification of cDNA.

PCR reactions were carried out in the presence of 1.5 m*M* $MgCl_2$, 200 μ*M* dNTP, 1.25 U of *Taq* DNA polymerase, 1 μ*M* primers and 2 μL of RT product according to Promega's PCR Core System II protocol. After 35 cycles (denaturation at 95°C for 30 s, annealing for 60 s at 50–55°C depending on the primers used, extension for 2 min at 72°C, and a final extension for 5 min at 72°C after the last cycle) of amplification, 5 μL of PCR reaction mix from a 50 μL total volume was fractioned in 1.5% agarose gel and stained with ethidium bromide. Negative controls were carried out in the absence of cDNA template to examine the cross contamination of samples.

PCR for β-actin was run for 25 cycles in parallel to rule out the possibility of RNA degradation or RNA transcription default. The primer sequences were designed to amplify the actin gene between exon 4 and exon 6 *(19)*: 5'GAGACCTTCAACACCCCAGC3' (sense); 5'GGACTCATCGTACTCCTGCTTG3' (antisense).

Sequencing of PCR Products

The RT-PCR products were extracted from agarose gel with Promega's Wizard™ PCR Preps DNA Purification System Kit, and the DNA sequence was determined by the dideoxy chain termination method using AmpliTaqs Cycle Sequencing Kit (Perkin-Elmer-Cetus).

Southern Blot Analysis of RT-PCR for GnRH-R

After electrophoresis, the GnRH-R PCR products were transferred onto Hybond-N^+ membrane (Amersham, Buckinghamshire, UK) using the Model 785 Vacuum Blotter from Bio-Rad according to the manufacturer's instructions. Hybridization was carried out in the presence of internal sequence oligonucleotide probes (see Fig. 1B) end labeled with [γ-^{32}P]-ATP using the DNA 5' End-Labelling System Kit from Promega. Hybridization was performed at 37°C overnight in the presence of 6X saline sodium citrate (SSC) (1X SSC = 0.15 *M* sodium chloride and 0.015 *M* sodium citrate), 2X Denhardt's solution (1 mg/mL of Ficoll, 1 mg/mL of polyvinylpyrrolidone, 1 mg/mL of bovine serum albumin), and 0.1% sodium dodecyl sulfate (SDS). The membrane was then washed three times (20 min each) in 2X SSC/0.1% SDS at room temperature and once at 50°C in 6X SSC/0.1% SDS for 30 min, and then scanned using the Storm 860 Gel and Blot Imaging System (Amersham Pharmacia Biotech) for autoradiographic intensity scanning.

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Reference

1. Sharoni, Y., Bosin, E., Miinster, A., and Schally, A. V. (1989). *Proc. Natl. Acad. Sci. USA* **86,** 1648–1651.
2. Manni, A., Santen, R., Harvey, H., Lipton, A., and Max, D. (1986). *Endocrinol. Rev.* **7,** 89–113.
3. Eidne, K. A., Flanagan, C. A., Harris, N. S., and Millar, R. P. (1987). *J. Clin. Endocrinol. Metab.* **64,** 425–432.
4. Emons, G., Pahma, G. S., Brack, C., Sturm, R., Oberheuser, F., and Knuppen, R. (1989). *Eur. J. Cancer Clin. Oncol.* **25,** 215–221.
5. Paz-Bouza, J. I., Redding, T. W., and Schally, A. V. (1987). *Proc. Natl. Acad. Sci. USA* **84,** 1112–1116.
6. Zalatnai, A. and Schally, A. V. (1989). *Cancer Res.* **49,** 1810–1815.
7. Greenway, B. A. (1987). *Br. J. Surg.* **74,** 441–442.
8. Fekete, M., Zalatnai, A., Comaru-Schally, A. M., and Schally, A. V. (1989). *Pancreas* **4,** 521–528.
9. Emons, G., Ortmann, O., and Becker, M. (1993). *Cancer Res.* **53,** 5439–5446.
10. Limonta, P., Dondi, D., Moretti, R.M., Fermo, D., Garattini, E., and Motta, M. (1993). *J. Clin. Endocrinol. Metab.* **76,** 797–800.
11. Wang, L., Xie, L. P., Huang, W. Q., Yao, B., Pu, R. L., and Zhang, R. Q. (2001). *Mol. Cell. Endocrinol.* **172,** 185–191.
12. Adelman, J. P., Mason, A. J., Hayflick, J. S., and Seeburg, P. H. (1986). *Proc. Natl. Acad. Sci. USA* **83,** 179–183.
13. Kaiser, U. B., Zhao, D., Cardona, G. R., and Chin, W. W. (1992). *Biochem. Biophys. Res. Commun.* **189,** 1645–1652.
14. Ohno, T., Imai, A., Furui, T., Takahashi, K., and Tamaya, T. (1993). *Am. J. Obstet. Gynecol.* **169,** 607–610.
15. Kakar, S. S., Grizzle, W. E., and Neill, J. D. (1994). *Mol. Cell. Endocrinol.* **106,** 145–149.
16. Gallagher, C. J., Oliver, R. T. D., and Oram, D. H. (1991). *Br. J. Obstet. Gynecol.* **98,** 1037–1041.
17. Szende, B., Zalatnai, A., and Schally, A. V. (1989). *Proc. Natl. Acad. Sci. USA* **86,** 1643–1647.
18. Huang, W. Q. and Zhang, C. L. (1998). *ACTA Anatomica Sinica* **21,** 299–301.
19. Nudel, U., Zakut, R., Shani, M., Levy, Z., and Yaffe, D. (1983). *Nucleic Acids Res.* **11,** 1759–1771.