Antisense Inhibition of Parathyroid Hormone-related Peptide Gene Expression Reduces Malignant Pituitary Tumor Progression and Metastases in the Rat

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ABSTRACT

A newly established metastatic rat pituitary tumor (mGH3) possesses a malignant phenotype that is invasive and hypervascular compared with the original GH3 tumors. mGH3 cells exhibit anchorage independence and expression of elevated levels of parathyroid hormone-related peptide (PTHrP) in vitro. To clarify the role of PTHrP in the development of the malignant phenotype, tumor cells were treated with phosphorothioate antisense PTHrP oligonucleotide. Treatment with antisense PTHrP resulted in a scattering phenomenon in the colony formation assay but did not inhibit cell growth in vitro. Inoculation of mGH3 cells in the cerebral ventricle resulted in a rapid growth of tumor cells within 3 weeks and dissemination throughout the entire ventricular system. Although treatment with sense or mismatched PTHrP oligonucleotide did not influence the subsequent tumor growth, the in vivo coinjection and injection of antisense PTHrP 1 week after tumor cell implantation into the right lateral ventricle markedly reduced tumor size and suppressed metastasis formation. The survival rate of mGH3 tumor-injected rats was prolonged by antisense PTHrP therapy. Our results demonstrated the biological involvement of PTHrP in malignant phenotype in rat pituitary tumors, suggesting that antisense PTHrP may provide a novel antimetastatic therapy for malignant somatotroph tumors.

INTRODUCTION

Several cytokine growth factors are essential for pituitary cell growth and function (1). The abnormal expression and/or regulation of these pituitary-derived peptides may be crucial for the development or maintenance of pituitary tumors. Endocrine tumors, including pituitary adenomas, are thought to possess common biological growth features associated only rarely with malignant transformation. Hallmarks of these tumors include relatively good clinical prognosis, hormone dependency, familial association, and only rare incidence of distant metastasis without local invasion (2). The molecular mechanism of multistep endocrine oncogenesis has been assessed, and several genetic mutations, including those for gsp, thyroid-stimulating hormone receptor, and ras and ret oncoproteins, have been reported recently (3–5). These abnormalities are related mainly to the development of adenomas but not to subsequent malignant transformation. Although several candidate oncogenes and antioncogenes are potentially involved in endocrine tumorigenesis, no critical molecular marker for advanced malignant transformation, including metastatic potential, has been identified (6), except for the p53 mutations described in the anaplastic change of thyroid papillary carcinoma (7), loss of retinoblastoma tumor suppressor gene, and allelic loss of the p53 gene in parathyroid carcinoma (8, 9).

PTHRP was originally characterized as a tumor product responsible for the humoral hypercalcemia of malignancy (10, 11). The PTHrP and PTHrP receptor genes were subsequently found to express in many normal and neoplastic tissues and physiologically function in both a paracrine and autocrine fashion (12, 13). PTHrP is commonly expressed in endocrine tumors (14, 15), and we also reported PTHrP expression in human pituitary adenomas and thyroid cancers (16, 17). In these previous studies, intense immunostaining for PTHrP was observed in spinal metastases of GH-secreting pituitary tumors and in the invasive components of pituitary tumors and thyroid carcinomas. Increased expression of PTHrP has also been observed in skeletal metastasis of human breast cancer (18). These results suggest that the expression of PTHrP is closely related to the malignant alteration of normal or adenoma endocrine cells. Furthermore, PTHrP is an essential factor in the cell growth of rat pituitary cell lines (19).

To elucidate the molecular mechanism of malignant transformation in pituitary tumors, we therefore focused on PTHrP expression and function in the mGH3 cell line derived from a lymph node metastatic deposit of a rat pituitary somatotroph adenoma cell line (GH3). Because pituitary tumors tend to invade the cerebral area (20), we further analyzed the in vivo malignant potential of mGH3 cell inoculation into the rat brain. This study examined the effect of PTHrP on pituitary tumor malignant transformation and demonstrated that antisense PTHrP oligonucleotide treatment attenuates in vivo tumor cell growth and metastases.

MATERIALS AND METHODS

Cell Lines

Rat pituitary somatotroph cell line GH3 (OGH3) cells were used (21). OGH3 cells were cultured in F-10 medium containing 10% FBS instead of 10% horse serum and 2.5% FBS culture conditions used in the original experiment (21). These OGH3 cells had reduced levels of GHF-1 and GH mRNAs (1). OGH3 cells in 10% FBS were implanted s.c. in Wistar-Furth female rats and yielded lymph node metastasis (2 of 25 tumor-bearing rats). Tumor cells from the lymph node metastatic sites were sequentially dispersed by enzymes (0.1% collagenase, 0.3% trypsin), transplanted, and passaged in vitro. The mGH3 cells were maintained in 2.5% FBS culture condition. Rat Leydig tumor cells producing PTHrP and osteoblast-like cells, ROS 17/2.8–5 expressing PTHrP/PTH receptor, were also cultured, as described previously (22).

Anchorage-dependent Cell Growth

Approximately 5 × 10^5 cells were plated in 35-mm tissue culture dishes in F-10 medium containing 15% horse serum and 2.5% FBS or 15% horse serum and 1% FBS. Cells were incubated at 37°C in a humidified atmosphere exposed to 5% CO₂-95% air-gas mixture. Cells were trypsinized and washed in Ca²⁺- and Mg²⁺-free PBS and diluted in a known volume of F10 medium containing trypan blue. The concentration of viable and dead cells in triplicate cultures was determined using a hemocytometer. For antisense experiments, 5 × 10^4 cells were plated with 10 μM PTHrP antisense or sense phosphorothioate oligonucleotide.

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Anchorage-independent Cell Growth (Soft Agar Colonization Assay)

Cells were trypsinized and washed in Ca²⁺- and Mg²⁺-free PBS, and 3 × 10² cells were plated in 1.0 ml of F-10 medium containing 1% or 10% FBS and 0.4% (w/v) top agar over 1.0 ml solid plug of F-10 medium with 0.6% agar. The size and number of colonies were counted after 14 days incubation. For antisense experiment, 10 μM PTHrP antisense or sense phosphorothioate was added simultaneously to the soft agar. Negative control studies were also performed in some experiments using the unrelated and mutated PTHrP oligonucleotides.

Northern Blot Analysis

Total RNA was extracted from oGH3 and mGH3 cells treated with either serum-free 10% FBS sense or antisense oligonucleotide by the acid guanidinium phenol method (23). Thirty mg of total RNA for each lane were electrophoresed on 1.5% agarose formamide gels. Transferred RNAs onto nylon membrane were hybridized in 50% formamide, 1× Denhardt’s reagent, 100 μg/ml denatured salmon sperm DNA, 5× SSC, and 0.1% SDS at 42°C overnight to a rat PTHrP cDNA probe (rPLPGH3, PvuII-XbaI insert) or rat PTH/PTHrP receptor cDNA (24) (kindly supplied by Drs. H. Juppner and G. V. Segre, Massachusetts General Hospital, Boston, MA) labeled with [32P]dCTP, according to the previously described random primer labeling methods (22). Blots were rehybridized to cyclophilin cDNA (25), and the relative density of PTHrP mRNA signals were corrected for cyclophilin. The differences in tumor size between groups were analyzed by ANOVA.

In situ hybridization

Using a digoxigenin RNA labeling kit (Boehringer Mannheim), antisense and sense RNA probes for rat PTH/PTHrP receptors were made from plpLP-R (H-N) reconstructed PTH/PTHrP receptor cDNA HindIII-NotI fragments into Bluescript vector. Hybridization was performed as described previously (17, 27). PTH/PTHrP receptor mRNA to be hybridized with digoxigenin-labeled riboprobe were detected by antibody to digoxigenin and stained 5-Bromo-4-chloro-3-indolylphosphate p-Toluidine Salt Nitroblue Tetrazolium Chloride (BGIP/NBT).

RESULTS

Establishment of mGH3 Cell Line. An in vitro cell line was derived from the lymph node metastasis of the rat pituitary somatotroph cell line (GH3). The oGH3 consisted of round-type cells with sparse growth (Fig. 1A) and showed the characteristics of benign pituitary tumor cells. Electron microscopy showed a round-shaped configuration with microvilli and small dense core granules (data not shown). In contrast, newly established mGH3 cells adhered to the culture flask and consisted of epithelial-like cells. A small number of these cells were multinucleated with reduced granularity (Fig. 1B). Neither cell line became confluent in the culture. mGH3 lymph node metastases developed 3 months after the s.c. implantation of GH3 cells. The tumor-bearing rats also demonstrated features of excessive secretion of GH; thus, the rats showed various acromegalic symptoms (e.g., soft tissue swelling; hypertension involving the ears, extremities, and tail; and generalized increased hair growth (data not shown)). Histological examination revealed that the tumor was invasive and hypervascular (Fig. 2, A and B).

Characterization of mGH3 Cells. Population-doubling time of oGH3 and mGH3 cells in 1% FBS was 42 ± 4.2 and 39 ± 3.9 h, respectively, and 35 ± 5.0 and 24 ± 4.5 h, respectively, in 2.5% FBS. The mGH3 cell growth rate in 2.5% FBS condition was significantly higher than that of oGH3 but not from that of 1% FBS condition. The mGH3 cell growth rate in 2.5% FBS condition was significantly higher than that of oGH3 but not from that of 1% FBS condition. Northern blot analysis indicated that PTHrP mRNA was not detected in oGH3 cells but was markedly overexpressed in mGH3 cells. PTHrP gene expression was, however, induced by pretreatment with FBS in both cell lines (Fig. 3). PTHrP receptor mRNA expression was not detected in both oGH3 and mGH3 cells by Northern blot.

Experiment 1. Tumor cells (1 × 10⁵; either oGH3 or mGH3) were implanted into the right lateral cerebral ventricle. The rats were killed 3 weeks later, and their tumors were fixed for histological examination and in situ hybridization.

Experiment 2. Tumor cells (1 × 10⁵) were inoculated into the cerebral ventricle with 10 μM PTHrP antisense or sense phosphorothioate oligonucleotide. After 3 weeks, the rat was killed, and the brain was fixed in 4% paraformaldehyde, cut into 3-mm-thick slices, and fixed overnight, followed by paraffin embedding. After hematoxylin and eosin staining, the growth of the developing tumor was assessed by measuring tumor areas using a microscopic image analyzer MCID (Fuji, Tokyo, Japan; control, n = 5; sense, n = 10; antisense, n = 10 (27)).

Experiment 3. PTHrP antisense or sense phosphorothioate oligonucleotides were injected into the cerebral ventricle 1 week after implantation of tumor cells. The extent of tumor growth and metastases were measured 3 weeks after implantation (sense, n = 10; antisense, n = 10) by the image analyzer.

In situ hybridization

Using a digoxigenin RNA labeling kit (Boehringer Mannheim), antisense and sense RNA probes for rat PTH/PTHrP receptors were made from plpLP-R (H-N) reconstructed PTH/PTHrP receptor cDNA HindIII-NotI fragments into Bluescript vector. Hybridization was performed as described previously (17, 27). PTH/PTHrP receptor mRNA to be hybridized with digoxigenin-labeled riboprobe were detected by antibody to digoxigenin and stained 5-Bromo-4-chloro-3-indolylphosphate p-Toluidine Salt Nitroblue Tetrazolium Chloride (BGIP/NBT).
analysis (Fig. 4). Immunohistochemical analysis also showed that PTHrP expression was markedly increased in mGH3 cells (data not shown). As a further test of the cell transforming capacity, cells were grown in double agar. The difference of the anchorage-independent colony formation between oGH3 and mGH3 cells was observed. Using 1% FBS, a clear colony formation by mGH3 cells was observed, but oGH3 cells did not form colonies. Using 10% FBS, colony formation was detected in both cell lines; however, enhancement of the colony size of mGH3 was more evident compared with that of oGH3 cells (mGH3 versus oGH3, 3.1 ± 0.6 mm versus 0.5 ± 0.2 mm; P < 0.001).

Effects of Antisense PTHrP Oligonucleotide Treatment on Anchorage-dependent and Anchorage-independent Cell Growth. To elucidate the direct effects of PTHrP on cell growth, PTHrP antisense
Suppression of mGH3 Tumorigenicity by Antisense Inhibition of PTHrP Gene Expression. As shown in Fig. 8, A and B, in vivo coinjection of mGH3 cells with antisense PTHrP oligonucleotide blocked tumor formation and dissemination of mGH3 cells. Tumor formation was virtually blocked for up to 4 weeks after a single oligonucleotide injection. The tumor tissue began to grow gradually afterward. The sense PTHrP oligonucleotide did not suppress tumor formation or dissemination. To investigate the time course of the observed antisense oligonucleotide effect, the antisense oligonucleotide was also injected 1 week after in vivo tumor cell inoculation. The 1-week delayed injection also suppressed tumorigenicity and relatively inhibited dissemination of the tumor into the subarachnoid space (Fig. 8, C and D). Infiltration of leukocytes or lymphocytes around the tumor was not observed. Tumor sizes among different treatment groups were determined using an image analyzer. Computerized morphometric analysis of coinjected oligonucleotide-treated groups revealed that the maximal cross-sectional area of the tumor was 35.04 ± 8.13 (control), 27.05 ± 10.59 (sense), and 0.68 ± 0.74 mm² (antisense). The tumor area of antisense-treated group was 50- and 40-fold smaller than that of the control and sense groups, respectively. An 8-fold inhibition of tumor growth was also observed in a group of 1-week interval injection of antisense PTHrP oligonucleotides (29.05 ± 10.59 mm²) in comparison with the sense treatment.

In Vivo Tumorigenicity. To further clarify the metastatic potential of mGH3 cells, intraventricular implantation by a stereobrain technique was performed. After intraventricular injection, mGH3 cells produced large tumors in the wall of the ventricle in a time-dependent fashion. Local invasiveness and dissemination to the third ventricle and basal lumens and abundant neovascularization occurred within 3 weeks of tumor cell inoculation. On the other hand, oGH3 cells seldom showed microscopic cell growth in the ventricular space (Fig. 7).

Northern blot analysis showed that treatment of cells with 10 μM antisense oligonucleotide for 72 h effectively suppressed PTHrP mRNA expression in mGH3 cells (Fig. 5). No difference in anchorage-dependent cell growth was observed with either sense or antisense treatment. Population-doubling time of mGH3 cells was 24.0 ± 4.5 h (no treatment), 26.2 ± 4.9 (sense treatment), and 25.2 ± 3.2 (antisense treatment), respectively, in 2.5% FBS. In testing anchorage-independent cell growth, the large mGH3 colonies became loose and formed satellite colonies after the antisense treatment (Fig. 6).

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Fig. 6. Colony formation assay of mGH3 cells in soft agar. Antisense (A) and sense (B) PTHrP oligonucleotides (10 μM) were added in top agar. After 2 weeks of incubation, colonies were observed by phase contrast microscopy. ×200.

group (3.62 ± 6.60 mm²; Fig. 9). The intensity of PTHrP positivity was markedly reduced in mGH3 tumors when treated with antisense PTHrP oligonucleotide compared with sense PTHrP treatment, even at 2 weeks after injection of the oligonucleotides (Fig. 10). Fig. 11 shows the effects of treatment with antisense, sense, or mismatched PTHrP oligonucleotides on the survival rates of the tumor-bearing rats. PTHrP antisense therapy significantly prolonged the survival of mGH3 tumor-bearing rats (control,

Fig. 7. In vivo tumorigenicity assay. Approximately 1 × 10⁶ mGH3 cells (A) and mGH3 cells (B) were implanted into the right lateral cerebral ventricle. After 3 weeks, histological examination was performed. Hematoxylin and eosin staining. ×20. Arrowheads, tumor cells.

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Fig. 8. Oligonucleotide treatment in vivo tumorigenicity assay. Oligonucleotide injected at the same time as tumor inoculation (A and B) or within 1-week interval after tumor inoculation (C and D). A and C, 10 μM antisense PTHrP oligonucleotide were injected. B and D, 10 μM sense PTHrP oligonucleotide were injected. Histological examination was performed 3 weeks after tumor implantation. Hematoxylin and eosin staining. ×20. Arrowheads, tumor cells.
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Control

Antisense

36.7 ± 4.9; sense, 38.1 ± 5.7; mismatched, 39.7 ± 6.9; antisense, 68.2 ± 14.3 days; \( P < 0.01 \).

Localization of PTH/PTHrP Receptor mRNA in mGH3 Tumors. To confirm the localization of PTH/PTHrP receptor mRNA in vivo in mGH3 tumor, in situ hybridization with antisense riboprobe of the PTHrP receptor gene was performed. The localization of PTH/PTHrP receptor mRNA was confirmed in only perivascular cells but not tumor cells themselves (Fig. 12). This finding supports the previous results of Northern blot analysis, which indicated the absence of PTH/PTHrP receptor mRNA in mGH3 cells.

DISCUSSION

The GH3 cell is a well-studied and established tumor cell line that secretes GH and prolactin. The cells originally exhibited characteristics of benign adenomas; however, no development of malignant pituitary or invasive phenotype occurred even after in vivo transplantation (21). The established mGH3 cells in this study possessed a highly invasive and metastatic phenotype, especially observed in vivo. mGH3 cells were confirmed to be of pituitary somatotroph cell origin because of the weak positive of a pituitary-specific transcriptional factor, GHF-1, and GH mRNAs (data not shown). Neovascularization, with involvement of the vascular endothelial cells, is also observed in mGH3 tumors. These findings indicated that a paracrine factor produced by mGH3 cells is necessary for the interaction with the surrounding normal cells or matrix during the process of in vivo growth and metastasis.

A comparison of the expression of c-myc or the antimetastatic gene *nm23* (28) in mGH3 cells with those of oGH3 cells indicated that these levels were not altered (data not shown). In contrast, a marked difference in PTHrP expression was observed during the GH3 malignant transformation. The colocalization of GH and PTHrP in a pituitary somatotroph tumor cell was also reported previously (15). Moreover, transgenic mice expressing GH-releasing factor yielded an increased coproduction of PTHrP and GH in a somatotroph cell. PTHrP was also a good marker of pituitary tumor progression in these transgenic mice (29). Although the *PTHrP* gene itself is not an oncogene or a tumor suppression gene, recent reports have described cellular transformation by the Ha-ras oncogene as being related to a dramatic increase in *PTHrP* gene expression (30) and activation of the metastatic potential of the cell (31). In some pituitary tumors, H-ras mutation has been described in metastatic lesions but not primary lesions (32). The response of the *PTHrP* gene may provide a crucial
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Epidermal growth factor that are known to stimulate PTHrP gene expression in some cell lines (33–35) and play an important role in tumor cell invasion and metastasis (36, 37). Therefore, to elucidate whether PTHrP expression is directly involved in malignant transformation or secondary phenomenon via another cytokine effect, we examined cell growth and colony formation in response to the antisense PTHrP oligonucleotide. Antisense inhibition of PTHrP gene expression had no effect on in vitro cell growth; however, the treatment exerted loosening of satellite colonies both in oGH3 cells treated with 10% FBS and in mGH3 cells, suggesting a weakening effect of antisense PTHrP oligonucleotide on cell-cell interactions. Recently, many factors such as adhesion molecules and matrix metalloproteinase have been demonstrated to influence the metastatic potential during the process of oncogenesis (38–40). Indeed, PTHrP is known to regulate cell adhesion and extracellular matrix proteins (41, 42). Additional studies are necessary to investigate how PTHrP may modulate cell motility or adhesion as a cytokine involved with malignant transformation.

We also examined the antisense inhibition of PTHrP in vivo because mGH3 demonstrated prominent in vivo tumorigenicity after intraventricular inoculation compared with the in vitro culture. These studies demonstrated a significant reduction of tumor cell progression and metastasis either after coinjection or within 1 week of injection of the antisense PTHrP oligonucleotide. Our data support in vivo long-term stability of phosphorylated oligomers for at least 1 week compared to unphosphorylated oligomers using in vivo rat amniotic fluid and in vitro culture medium (data not shown). Although the PTH/PTHrP receptor mRNA was not detected in mGH3 by Northern blotting analysis, PTHrP receptor mRNA was detected in perivascular cells around mGH3 cells but not in tumor cells by in situ hybridization.

Because the pitfalls of antisense oligonucleotides are well known,
we have used a mismatched PTHrP oligonucleotides and other unre- 
lated genes (IGF-I or scrambled). The oligonucleotides for other 
genes were not effective in suppressing mOH3 tumor formation (data 
not shown). As the immune action by unmethylated CpG deoxyoli-
gonucleotides has been reported to have implications for the design 
and interpretation of studies using antisense oligonucleotides (43), the 
precise mechanism of in vivo effect of antisense PTHrP remains 
to be further clarified. However, the number of CpG deoxyoligo-
gonucleotides is the same among the various oligonucleotides used 
in these experiments.

Although the keratinocyte cell line is enhanced in in vitro cell growth 
by antisense RNA for PTHrP (44), this study shows that endogenous 
PTHRP plays a crucial role in pituitary tumor cell proliferation, closely 
related to malignant transformation in a paracrine fashion. It was of 
interest that disruption of PTHrP causes death in mice, showing a marked 
reduction of growth plate and manifestation of apoptosis, leading to local 
calcification during the developmental stage of enchondral bone (45). 
These findings also may support the notion of a paracrine action of 
PTHRP. On the other hand, the antisense action of colony formation assay 
has indicated that a different type of PTHrP-specific receptor may be 
involved in the autocrine regulation of mGH3 cell tumor growth and 
motility. Although the precise mechanism of the effect of antisense 
PTHRP oligonucleotide on mGH3 tumor growth is still unclear, the 
observed tumor-suppressive effect is probably because of the bifunctional 
in vivo action of PTHrP on pituitary tumor cell growth via paracrine and 
autocrine mechanisms. Recent evidence also supports the differences 
between the effects observed in vivo versus in vitro using antisense genes 
(46, 47).

Successful tumor immunotherapy using sense or antisense expres-
sion vectors has been recognized recently (48, 49). Effective antisense 
oligonucleotide therapy to reverse metastatic transformation has been 
developed with nuclear factor κB antisense inhibition in fibroblast 
tumors of T-cell leukemia virus Tax gene transgenic mice (50), and 
therapeutic option for inoperable invasive pituitary somatotroph tumors 
by inhibiting cell migration and neovascularization.

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