Menin Inactivation Leads to Loss of Transforming Growth Factor β Inhibition of Parathyroid Cell Proliferation and Parathyroid Hormone Secretion

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ABSTRACT

Primary hyperparathyroidism is a common endocrine disorder caused by parathyroid gland enlargement and excessive parathyroid hormone (PTH) secretion. However, the precise mechanisms of tumorigenesis of the parathyroids are unknown. Here we have investigated the roles of transforming growth factor (TGF)-β and menin, the product of the multiple endocrine neoplasia type 1 (MEN1) gene, in the proliferation and PTH production of parathyroid cells from patients with secondary hyperparathyroidism or Men1. TGF-β was expressed in the parathyroid endocrine cells. Addition of TGF-β to parathyroid cells from patients with secondary hyperparathyroidism inhibited their proliferation and PTH secretion. These responses to TGF-β were lost when menin was specifically inactivated by antisense oligonucleotides. Moreover, TGF-β did not affect the proliferation and PTH production of parathyroid cells from a Men1 patient. These results indicate that menin is required for TGF-β action in the parathyroid. We conclude that TGF-β is an important autocrine/paracrine negative regulator of parathyroid cell proliferation and PTH secretion and that loss of TGF-β signaling due to menin inactivation contributes to parathyroid tumorigenesis.

INTRODUCTION

Primary hyperparathyroidism is a common disorder characterized by hypercalcemia caused by an excessive secretion of parathyroid hormone (PTH). This is due to an increased mass of one or more parathyroid gland and a resetting of the control of PTH secretion from the parathyroid endocrine cells by the ambient calcium concentration. Previous studies have implicated altered proto-oncogenes and tumor suppressor genes in parathyroid tumorigenesis (1). For example, overexpression of the proto-oncogene cyclin D1 is related to the development of parathyroid adenoma (2, 3), whereas loss of chromosome 13q, which contains the retinoblastoma and other potential tumor suppressor genes, is related to parathyroid carcinoma (4). However, the precise mechanisms of tumorigenesis in the parathyroid are not yet clear.

Multiple endocrine neoplasia type 1 (MEN1; 131100, Mendelian Inheritance in Man (MIM)) is a cancer predisposition syndrome inherited as a dominant trait. It affects a variety of endocrine tissues, in particular, parathyroids, endocrine pancreas, anterior pituitary, foregut-derived neuroendocrine tissues, and adrenal cortex. The MEN1 gene, on chromosome 11q13 (5), encodes a 610-amino acid protein called menin (6, 7), and more than 300 independent germ-line and somatic mutations scattered throughout the protein-coding region have been identified (8, 9). Somatic mutations have been found in the corresponding sporadic endocrine tumors, for example, biallelic inactivating Men1 defects occur in up to 20% of sporadic parathyroid adenomas (10–12). Many of the mutations are clearly inactivating, giving rise to a truncated product. Consequently, lack of menin caused by loss of both alleles leads to tumor development, consistent with menin acting as a tumor suppressor. Furthermore, stable overexpression of menin in ras-transformed NIH 3T3 cells inhibits cell growth and tumor formation in nude mice (13), and mice with heterozygous menin inactivation develop endocrine tumors, including those of the parathyroid, later in life and thus exhibit a phenotype similar to that of the human MEN1 disorder (14, 15). Although there is some evidence that menin function may be related to transcriptional regulation and cell cycle control and that menin interacts with a variety of protein partners (16–19), the mechanisms by which menin inactivation causes endocrine tumorigenesis and its physiological functions are not known.

It is known that transforming growth factor (TGF)-β causes growth inhibition of many different cell types, and genes encoding proteins that are essential for the TGF-β signaling pathway are inactivated in several cancers (20, 21). We demonstrated previously that menin is implicated in TGF-β signaling and interacts with Smad3, a crucial mediator of the TGF-β signaling pathway, in pituitary cells (16). In the present study, we have examined the roles of TGF-β and menin in the proliferation and PTH production of human parathyroid cells.

MATERIALS AND METHODS

Chemicals. Human recombinant TGF-β was from Sigma (St. Louis, MO), anti-proliferating cell nuclear antigen (PCNA) antibody was from R&D Systems, Inc. (Minneapolis, MN), and anti-TGF-β antibody was from Transduction Laboratories (Lexington, KY). A polyclonal antibody against the NH2 terminus of human PTH was from Chemicon (Temecula, CA). The goat polyclonal antibody against the COOH terminus of human menin was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The menin rabbit polyclonal antibody was raised against a decapentapeptide (synthesized by solid-phase chemistry at the Peptide Synthesis Facility of the Sheldon Biotechnology Centre of McGill University) corresponding to amino acids 476–489 of menin as described previously (18). Twenty-four-bp phosphorothioate-derivatized antisense and sense menin oligonucleotides were synthesized on an automated solid phase synthesizer (Oligonucleotide Synthesis Facility of the Sheldon Biotechnology Centre of McGill University) by standard phosphoramidite chemistry. The sequence of antisense oligonucleotide was 5′-GGCCTTCACGCCCCATGGGGGCGGCGGG-3′, and the sequence of sense oligonucleotide was 5′-CCCCCGCCCATGGGGGCTGAAG-3′. All chemicals used were of analytical grade.

Cell Culture. Cells were prepared from hyperplastic parathyroid glands removed surgically from uremic hemodialysis patients and a patient with MEN1. Approval from the local ethical committee and informed consent were obtained. Tissues were minced into small fragments and digested at 37°C with collagenase type II (Wako, Osaka, Japan) in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with trypsin-glutamine and without sodium bicarbonate. The parathyroid tissue fragments were mechanically dispersed by aspiration every...
performed using an unpaired t test. The resulting pellet was washed with serum-free RPMI 1640 and resuspended in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies, Inc.). After trypsin blue exclusion test for cell viability, the dispersed cells were distributed into tissue culture dishes or chamber slides (Lab Tek, Naperville, IL) and incubated in a humidified 95% air/5% CO2 atmosphere at 37°C for 4 days.

**Immunoblot Analysis.** Cells were lysed with a radioimmunoprecipitation buffer containing 0.5 mM phenylmethylsulfonyl fluoride, complete protease inhibitor mixture, 1% Triton X-100, and 1 mM sodium orthovanadate. Cell lysates were centrifuged at 12,000 × g for 20 min at 4°C, and the supernatants were stored at −80°C. Protein quantitation was performed with the BCA protein assay reagent (Pierce, Rockford, IL). Protein aliquots were denatured in SDS sample buffer and separated on 10% polyacrylamide-SDS gels. Proteins were transferred in 25 mM Tris, 192 mM glycine, and 20% methanol to polyvinylidene difluoride membranes. Blots were blocked with TBS (20 mM Tris-HCl (pH 7.5) and 137 mM NaCl) plus 0.1% Tween 20 containing 3% dried milk powder. The antigen-antibody complexes were visualized using appropriate secondary antibodies (Sigma), and the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) as recommended by the manufacturer. For all experiments, 20 μg of protein were applied to each gel lane.

**Immunohistochemistry and Immunocytochemistry.** For immunohistochemistry, formalin-fixed and paraffin-embedded specimens were deparaffinized with xylene and rehydrated through a series of graded alcohols, whereas for immunocytochemistry, the primary cultured parathyroid cells in chamber slides were fixed in 4% formalin for 10 min. After blocking endogenous peroxidase activity with 0.3% H2O2 in methanol, the sections were incubated with the specific first antibody in a humidified chamber at room temperature for 1 h. The avidin-biotin-peroxidase complex method was used with the Dako Lab kit (Dako, Carpinteria, CA). Final development of the sections was carried out with 3,3’-diaminobenzidine containing 0.03% H2O2.

**[3H]Thymidine Incorporation Assay.** Parathyroid cells were seeded at 2 × 10^5 cells/well in 24-well plates and maintained in RPMI 1640 with 10% fetal bovine serum. After 48 h of culture, cells were labeled with 0.5 μCi/ml [3H]thymidine (Amersham Pharmacia Biotech, Bucks, United Kingdom) for 24 h. The medium was removed, cells were washed twice with PBS, and 5% trichloroacetic acid was added on ice for 10 min. After removal of the trichloroacetic acid, the residue was dissolved in 20 mM NaOH at 37°C. Scintillation mixture was added to each sample, and radioactivity was counted in a liquid scintillation counter.

**The 3-(4-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Dye Assay.** Mitochondrial function was assayed by the ability of viable cells to convert soluble MTT dye (Sigma) into an insoluble dark blue formazan reaction product. MTT dye was dissolved in PBS at a concentration of 5 mg/ml and sterilized by passage through a 0.22-μm filter. This stock solution was added (1 part:10 parts medium) to each well of a 96-well tissue culture plate, and the plate was incubated at 37°C for 12 h. Acid isopropanol (400 μl of 10 μl HCl in 100 ml of isopropanol) was added to each well and mixed thoroughly to ensure that all of the crystals were dissolved. The plates were read on a microplate reader at a wavelength of 595 nm.

**Measurement of PTH Secretion and MEN1 Gene Mutation Analysis.** Cells were cultured in 6-well plates in supplemented medium until subconfluence, pretreated with antisense or sense oligonucleotides, and then treated with TGF-β1. After a 24-h incubation, PTH in the medium was measured by radioimmunnoassay (PTH Kit, Yamasa Corp., Choshi, Japan), and the results were expressed in pg/ml (22). Men1 gene mutation analysis was performed as described previously (23).

**Statistics.** Data are expressed as mean ± SE. Statistical analysis was performed using an unpaired t test or ANOVA.

**RESULTS**

**TGF-β in Parathyroid Endocrine Cells.** By immunoblot analysis, TGF-β was detected in cultured parathyroid cells from patients with secondary hyperparathyroidism (Fig. 1A). By immunohistochemistry, PTH was detected in the parathyroid endocrine cells, but not fibroblasts (Fig. 1B). TGF-β was also detected in the parathyroid endocrine cells, but it was not clearly detected in the other cells, such as the surrounding fibroblasts (Fig. 1C). Similar findings were obtained in parathyroid tissues from patients with primary hyperparathyroidism (data not shown). These results show that TGF-β is expressed mainly in the parathyroid endocrine cells and suggest that TGF-β could regulate parathyroid cell function in an autocrine or paracrine manner.

**Effects of TGF-β on the Expression of Menin.** TGF-β increased the expression of menin in cultured parathyroid cells in a time-dependent manner as assessed by immunoblot analysis (Fig. 2A). Increased menin expression occurred within 1 h of TGF-β stimulation and reached a maximum at 6 h. These results indicate that TGF-β modulates the expression of menin in parathyroid cells and raised the possibility that menin is important for TGF-β functions in the parathyroid. To assess the effects of menin inactivation, we used antisense menin oligonucleotides. For the negative control, we used sense menin oligonucleotides. Antisense oligonucleotides effectively inhibited endogenous menin in primary cultures of parathyroid cells, whereas sense oligonucleotides had no effect (Fig. 2B). These findings suggest that antisense oligonucleotides effectively and specifically depleted endogenous menin in parathyroid cells.

**TGF-β Inhibits the Proliferation of Parathyroid Cells.** We next tested the effects of TGF-β on the proliferation of cultured human
Menin expression was assessed by immunoblotting as described in “Materials and Methods.” As a control for protein loading, blots were probed with a β-actin antibody.

Parathyroid cells from a patient with secondary hyperparathyroidism were cultured for 12 h in the absence (Cont.) or presence of antisense (AS) or sense (S) menin oligonucleotides. Menin expression was assessed by immunoblotting as described in “Materials and Methods.” As a control for protein loading, blots were probed with a β-actin antibody.

Because menin is important for the proliferation of parathyroid endocrine cells, we hypothesized that menin might serve a similar function in parathyroid cells. We therefore examined the effects of menin inactivation by antisense oligonucleotides on the TGF-β-mediated reduction in proliferation of primary cultures of parathyroid cells with [3H]thymidine incorporation, MTT dye assay, and PCNA immunoblot and immunocytochemistry analyses. Antisense oligonucleotides antagonized the TGF-β-mediated reduction in cell proliferation, whereas sense oligonucleotides had no effect (Fig. 3, A, C, and D). These results show that menin inactivation antagonizes the TGF-β-mediated growth inhibition of parathyroid cells.

Effects of TGF-β on PTH Production and Secretion. Excessive PTH secretion is characteristic of parathyroid tumors. We therefore examined the effects of TGF-β on PTH expression in primary cultured parathyroid cells. The number of PTH-positive cells relative to the total number of parathyroid cells was >90% (Fig. 4A, i). TGF-β decreased the number of PTH-positive cells to 40% relative to the vehicle-treated group (Fig. 4A, i and ii; Fig. 4B). By immunoblot analysis, the expression of PTH was detected as molecular species of 10 kDa and smaller (Fig. 4C). The intensity of these bands was reduced with TGF-β (Fig. 4C, Cont.). Next, we examined the level of

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**Fig. 2.** Effects of transforming growth factor β and antisense menin oligonucleotides on parathyroid menin. A, parathyroid cells from a patient with secondary hyperparathyroidism were cultured with 2.5 ng/ml TGF-β for the indicated times, and menin expression was assessed by immunoblotting as described in “Materials and Methods.” B, parathyroid cells from a patient with secondary hyperparathyroidism were cultured for 12 h in the absence (Cont.) or presence of antisense (AS) or sense (S) menin oligonucleotides. Menin expression was assessed by immunoblotting as described in “Materials and Methods.” As a control for protein loading, blots were probed with a β-actin antibody.

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**Fig. 3.** Effects of transforming growth factor (TGF)-β and menin on parathyroid cell proliferation. A, parathyroid cells from a patient with secondary hyperparathyroidism were cultured without (Cont.) or with antisense (AS) or sense (S) menin oligonucleotides for 6 h. Cells were then cultured in fresh media without or with antisense or sense oligonucleotides without (vehicle) or with 2.5 ng/ml TGF-β for an additional 48 h, and [3H]thymidine incorporation was measured as described in “Materials and Methods.” Values are the mean ± SE of triplicate determinations. *P < 0.01 compared with the corresponding TGF-β-treated group. B, parathyroid cells, as described in A, were cultured without (Cont.) or with increasing concentrations of antisense (AS) menin oligonucleotides for 6 h. Cells were then cultured in fresh media without or with antisense oligonucleotides without (vehicle) or with 2.5 ng/ml TGF-β for 72 h, and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye assay was performed as described in “Materials and Methods.” Each value is the mean ± SE of triplicate determinations. *P < 0.01 compared with the TGF-β-treated, antisense-untreated group. C, parathyroid cells, as described in A and B, were cultured in chamber slides either without (–) or with (+) antisense (AS) or sense (S) menin oligonucleotides for 6 h. Cells were then cultured in fresh media without or with antisense or sense oligonucleotides in the absence (–) or presence (+) of 2.5 ng/ml TGF-β for 24 h, and proliferating cell nuclear antigen immunocytochemistry and counterstaining with methyl green were performed as described in “Materials and Methods.” Representative views of cells cultured (i) without TGF-β or oligonucleotides, (ii) with TGF-β and sense oligonucleotides, or (iii) with TGF-β and antisense oligonucleotides are shown. Magnification factor, ×200. D, proliferating cell nuclear antigen-positive cells for each group described in C. Each value is the mean ± SE relative to the vehicle-treated control group. *P < 0.01 compared with the corresponding TGF-β-untreated group.
PTH in the culture medium. At baseline, the level of PTH was 310 ± 3.93 ng/ml (Fig. 4D). In cells treated with TGF-β, the level of PTH was significantly less at 170 ± 2.52 ng/ml, 55% of that of cells not treated with TGF-β (Fig. 4D, Cont.). These results show that TGF-β inhibits the production of PTH in and its secretion from parathyroid cells.

**Effects of Menin Inactivation on PTH Production.** Next, we examined the effects of menin inactivation with antisense oligonucleotides on PTH expression in the cultured parathyroid cells. Antisense oligonucleotides antagonized the TGF-β-mediated reduction in the number of PTH-positive cells (Fig. 4, A and B), the cellular expression of PTH (Fig. 4C), and the level of PTH in the medium (Fig. 4D). On the other hand, the control sense oligonucleotides did not affect TGF-β-induced inhibition of PTH production and secretion. Furthermore, antisense oligonucleotide treatment increased the basal level of PTH expression and secretion (for example, see Fig. 4D, vehicle alone, AS versus Cont.). Together, these findings show that menin inactivation not only antagonizes the TGF-β-mediated reduction in PTH production of the parathyroid cells but actually stimulates the basal level of PTH.

**Men1 Mutation and Menin Expression in a Patient with MEN1.** Direct sequence analysis of PCR-amplified Men1 exon 2 from leukocyte DNA of a MEN1 patient identified a heterozygous mutation (CGA→TGA) changing codon 29 from arginine to a stop codon (R29X; Fig. 5A). Analysis of the patient’s parathyroid tumor DNA demonstrated loss of the wild-type allele (loss of heterozygosity). Immunohistochemistry of normal parathyroid tissue demonstrated menin expression (Fig. 5B); however, a similar analysis failed to identify menin expression in the parathyroid tumor of the MEN1 patient (Fig. 5C). Therefore, consistent with Knudson’s two-hit hypothesis (24), the parathyroid tumor from this patient lacked functional menin.

**Effects of TGF-β on Proliferation and PTH Production in Parathyroid Cells of a MEN1 Patient.** We next examined the effects of TGF-β on the proliferation and PTH expression in parathyroid cells from the MEN1 patient. With TGF-β treatment, the number of PCNA-positive parathyroid cells from the MEN1 patient were not altered significantly (Fig. 6A). This is in marked contrast to the effect of TGF-β in reducing the number of PCNA-positive cells from patients with secondary hyperparathyroidism (Fig. 3D). These findings indicate that the inhibitory effect of TGF-β on proliferation has been lost in the parathyroid cells from the MEN1 patient. With respect to PTH production and secretion, TGF-β treatment did not affect the number of PTH-positive cells and the medium PTH levels (Fig. 6, B and C). Similar findings were found in cultured parathyroid cells from a second MEN1 patient (data not shown). These results indicate that menin inactivation antagonizes the TGF-β-mediated reduction in proliferation and PTH production in human parathyroid cells.

**DISCUSSION**

Several local growth factors such as endothelin-1, TGF-α, fibroblast growth factors, and insulin-like growth factors are expressed in parathyroid cells and in some cases have been shown to stimulate the proliferation of parathyroid cells (25–31). TGF-β is produced in many tissues and normally functions as a local negative growth regulator, although its expression in parathyroid gland and its role in parathyroid cell function have not been examined previously. The present study
shows that TGF-β is expressed in the parathyroid, predominantly by the endocrine cells, and that TGF-β negatively regulates the proliferation and PTH production of these cells.

TGF-β and components of its signaling pathway that mediate its function as a negative growth regulator can be considered as tumor suppressors. The blocking of TGF-β signaling may disrupt the delicately balanced cellular steady state, pushing the cell toward inappropriate growth that eventually results in tumor formation. Indeed, several reports have described inactivating mutations in genes encoding proteins known to be essential for the TGF-β signaling pathway in a variety of cancers (20, 21). Experiments in mice have provided additional evidence for the role of TGF-β in protection against increased cellular proliferation and tumor development in response to hormones or carcinogens (32–34). There is evidence that oncoproteins abrogate normal cellular growth control by blocking the TGF-β signaling system, and we reported previously that inactivation of a tumor suppressor gene, menin, causes derangement of the TGF-β signaling pathway in pituitary cells (16). The present findings support the notion of TGF-β as a local growth inhibitory regulator, which functions as a tumor suppressor, inhibiting deregulated cell proliferation leading to tumorigenesis and tumor progression.

Deregulated PTH production is another important characteristic of parathyroid tumors. Several reports have indicated that TGF-β inhibits hormone secretion in endocrine cells such as those of the pituitary (35–37). The present study is the first to show that TGF-β inhibits the production and secretion of PTH in parathyroid cells. The pathological severity of hyperparathyroidism is a result of the overall production of PTH that depends on secretory responsiveness as well as the mass of the tumor. The ambient calcium concentration monitored by the calcium-sensing receptor, the ambient phosphate concentration sensed by the parathyroid in an unknown fashion, and 1,25-dihydroxyvitamin D interacting through the nuclear vitamin D receptor are crucial regulators of parathyroid growth and secretory function. In future studies, it will be important to examine if and how TGF-β interacts with these regulatory factors in modulating parathyroid function.

A role for alterations in the Men1 tumor suppressor gene has been clearly established in the pathogenesis of sporadic primary parathyroid tumorigenesis. Specific clonal alterations involving somatic mutation and/or deletion of both Men1 alleles have been demonstrated in up to 20% of sporadic adenomas (10). Allelic losses on 11q occur in roughly twice this number of adenomas. Thus, mutations in regions of the Men1 gene not presently examined, for example, gene regulatory regions and introns, may be involved in these cases. It had been assumed that primary parathyroid hyperplasia and uremic refractory secondary hyperparathyroidism involved polyclonal, nonneoplastic cellular proliferation. However, the monoclonal nature of a substantial number of the “hyperplastic” tumors from such patients has been demonstrated by X chromosome inactivation analysis (38). In addi-
tion, allelic loss of chromosome 11 markers and/or Men1 gene inactivation has been demonstrated in only a very few of these types of tumor (39–43). Thus, a Men1 gene abnormality rarely plays a role in the clonal emergence in uremic parathyroid hyperplasia. Given this background, in the present study we have used tissue from uremic hyperparathyroid patients as an appropriate surrogate for normal human parathyroid tissue in which the Men1 gene is not impaired.

We hypothesized that menin might be involved in the growth inhibitory actions of TGF-β in parathyroid cells. We first showed that the expression of menin was induced rapidly by TGF-β in primary cultured parathyroid cells, similar to the effect of TGF-β in pituitary cells (16). We then showed that menin inactivation antagonized the TGF-β-induced inhibition of proliferation and PTH expression in parathyroid cells. These data show that menin is critical for the TGF-β pathway in parathyroid cells and suggest that the loss of function of this pathway may be critical to the role of menin inactivation in parathyroid tumorigenesis.

Several lines of evidence suggest a relationship between menin and hormone production in endocrine cells. Menin inhibits insulin production and prolactin gene promoter activity in rat insulinoma and pituitary tumor cell lines, respectively (44, 45). Indeed, the present study shows that decreased menin expression, achieved by the antisense oligonucleotide method, is associated with enhanced PTH production in human parathyroid cells. Also, menin is clearly required for the TGF-β-induced reductions in PTH production and secretion because in both parathyroid cells from uremic secondary hyperparathyroid patients treated with menin antisense oligonucleotides and parathyroid cells from a patient with MEN1, PTH synthetic and secretory responses to TGF-β were impaired. Mouse models of MEN1 have been generated by ablation of the Men1 gene. In one case, although heterozygous null mice developed parathyroid tumors during their lifetime, biochemical hyperparathyroidism was not documented (14). Another Men1 heterozygous knockout strain developed the same range of endocrine tumors as seen in Men1 patients, including those of the parathyroid, and elevated PTH levels were observed in these mice (15). In a third example, a mouse model having homozygous parathyroid gland-specific deletion of the Men1 gene developed parathyroid neoplasia and hypercalcemia, although circulating PTH levels were not measured (46). Clearly, our study directly shows that menin is important for basal and regulated PTH production.

In conclusion, our results indicate that TGF-β is a critical negative regulator of parathyroid cell proliferation and PTH secretion and that loss of TGF-β signaling due to menin inactivation likely contributes to parathyroid tumorigenesis. This important role of TGF-β suggests it as a novel therapeutic target for hyperparathyroidism. This would be most relevant to secondary hyperparathyroidism in which derangements of the TGF-β/Smad pathway due to menin mutations are rarely implicated in the development of hyperplasia.

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