Parathyroid Neoplasia and Hypercalcemic Hyperparathyroidism

The inactivation of the MEN1 tumor suppressor gene in patients leads to a constellation of changes in endocrine tissues, including parathyroid neoplasia, pituitary adenomas, pancreatic neuroendocrine tumors, and carcinoids. To study the pathophysiological consequences of the deletion of the MEN1 gene, we set out to create a mouse model of hyperparathyroidism resulting from the deletion of the Men1 gene in parathyroid tissue. We introduced a Men1 gene flanked by loxP sites into the mouse germ line and then used a parathyroid cell-specific promoter to drive the expression of Cre recombinase, resulting in the deletion of the Men1 gene. Here, we show that loss of Men1 gene function in the parathyroid glands of mice results in histological changes consistent with parathyroid neoplasia as well as systemic hypercalcemia. This model provides a means for dissecting the molecular basis of this familial cancer syndrome and may allow for the development of new strategies to treat related forms of hypercalcemia.

INTRODUCTION

MEN1 [Online Mendelian Inheritance in Man (OMIM) 131100] is an autosomal dominant familial cancer syndrome resulting from the heterozygous inactivation of the MEN1 tumor suppressor gene (1, 2). Patients develop tumors affecting all four parathyroid glands, multiple neuroendocrine tumors of the pancreas including gastrinomas and insulinomas, pituitary adenomas, as well as a variety of other manifestations including foregut carcinoids and skin lesions (2, 3). Because the MEN1 gene was cloned and its protein product, menin, was identified (4), there has been a concerted effort to develop models to better understand the function of menin and the role its loss plays in the pathogenesis of MEN1-related tumors. The MEN1 gene seems to function as a classic tumor suppressor; somatic loss of the remaining wild-type allele is a necessary step in tumorigenesis in MEN1 tumors or in sporadic neoplasms.

Our group has reported previously that a traditional knockout mouse model of the Men1 gene results in embryonic lethality for mice homozygous for the deletion, whereas heterozygous knockout mice develop tumors highly reminiscent of the human MEN1 syndrome after 12 months of age (5). Although mice with heterozygous Men1 inactivation developed histological evidence of parathyroid neoplasia, hypercalcemia was not observed (5). Evidence for hyperparathyroidism on pathological examination of parathyroid glands has also been seen in heterozygous Men1 mutant mice by others, although hypercalcemia was not reported (6). To study more precisely the consequences of homozygous deletion of the Men1 gene in somatic tissues and to determine whether this genetic alteration would result in a model for primary hyperparathyroidism, we developed a strategy for the tissue-specific deletion of the Men1 gene in the parathyroid glands of mice using site-specific DNA deletion (7–9).

MATERIALS AND METHODS

DNA Constructs

PTH-Cre. The 5′ upstream regulatory region of the human PTH gene was cloned into pBluescript SK using the SacI and BglII sites in the multiple cloning region. The Cre recombinase cDNA (with a metallothionein-I polyadenylation signal) was placed downstream of the human PTH promoter by excising Cre from pBS185 (Life Technologies, Inc.) using a Xhol and HindIII digest and using a blunt ligation into the downstream BglII site. Correct orientation of the insert was confirmed with restriction digest and partial sequencing.

Floxed Men1 (dNdN). Mice were generated that contained inserted loxP sites in introns 2 and 8 of the Men1 gene by breeding the existing line, Men1/TS/G1E +/+ (5), with EIIa-Cre mice that express Cre ubiquitously from the EIIa promoter (10). This breeding resulted in conditionally targeted mice for Men1 that were bred to homozygosity and termed Men1dNdN mice.

Microinjections

The 9.9-kb DNA fragment was isolated from the PTH-Cre vector by restriction digest, and DNA was purified and prepared for microinjection using standard techniques in the National Institute of Diabetes and Digestive and Kidney Diseases Transgenic Facility.

The generation of mice carrying the floxed Men1 gene has been described previously (5).

Animal Handling

All animals were treated and maintained in accordance with NIH and Association for Assessment and Accreditation of Laboratory Animal Care guidelines under approved National Institute of Diabetes and Digestive and Kidney Diseases Institutional Animal Care and Use Committee protocols.

The mice were housed with a photoperiod of 12 h light/12 h dark. Their standard diet was Ziegler Rodent NIH-31 Open Formula, which contained 1.11% calcium, 0.93% phosphorus, and 4.0 IU/g vitamin D3. The feeding trough was filled biweekly with 400 g of rodent food/cage housing no more than five mice. Blood was collected by tail nicking. Serum was stored at –20°C until assayed for serum calcium concentration.

Screening of Transgenic Mice

Genomic DNA was isolated from the tips of mouse tails by incubation with proteinase K (250 μg/ml) overnight at 50°C with shaking. The DNA was extracted with phenol/chloroform and quantified by spectrophotometry. Approximately 0.1 μg of genomic DNA underwent multiplex PCR (95°C for 1 min; 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min for 35 cycles; 72°C for 10 min; 4°C hold) using three primers for the detection of wild-type and floxed Men1 DNA. Primer A (CCCATCGTCCCTCTCCTGATG) is specific to exon 1 of the Men1 gene, and primer B (CCCTCGGGTTATTCACCC- GCAGG) is specific for the wild-type DNA sequence, which is deleted in cloning the floxed Men1 construct. Primer C (CCGAGGAAAGGTACTT-
GAAATGGC) is specific for the inserted floxed sequence. The combinations of primers A and B yield a wild-type 300-bp amplicon, and primers A and C yield a 236-bp targeted amplicon. For detecting the presence of Cre recombinase DNA, genomic DNA underwent PCR (95°C for 1 min; 95°C for 30 s, 65°C for 1 min for 35 cycles; 72°C 10 min; 4°C hold) with primer CreF (ACCTGAAGATGTTCGCGATTATCT) and primer CreR (ACCGTCAGTACGTGAGATATCCTT). The presence of Cre recombinase results in a 450-bp amplicon. LacZ-positive mice were detected by PCR (95°C for 1 min; 95°C for 30 s, 65°C for 30 s, and 72°C for 1 min for 35 cycles; 72°C 10 min; 4°C hold) with LacZ F (GATCCGCGCTGGCTACCGGC) and LacZ R (GGATACTGACGAAACGCCTGCC) primers, with the presence of LacZ DNA resulting in a 350-bp amplicon. Southern blotting analyses confirmed the genotype expression of the various transgenic mouse lines.

**Crosses to Reporter Strains**

PTH-Cre-positive mice were crossed with Z/AP reporter mice (11), and the progeny was screened by tail snip and PCR. PTH-Cre-positive and Z/AP-positive mice were then sacrificed, and tissues were harvested and snap frozen in liquid nitrogen. The frozen tissue was stored at −80°C in OCT (Tissue Tek; Sakura), cryosectioned at 10 μm on charged slides, and fixed in 0.2% glutaraldehyde for 1 h. For alkaline phosphatase and β-gal staining, sections were processed as described previously (12). Briefly, for alkaline phosphatase staining, slides were fixed in 0.2% glutaraldehyde for 1 h. Endogenous alkaline phosphatase was inactivated by incubating slides in PBS at 75°C for 30 min, washed, and overlayed with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate stain (Boehringer) for 12 min in the absence of light.

**Crosses between PTH Cre-positive and Floxed Men1 Mice**

Mice found to be PTH-Cre positive (Friend Virus B strain) were crossed with homozygous floxed Men1-positive mice (129SvEvTac). Progeny were screened by tail snip as described above, and additional matings were arranged based on genotype.

**Determinations of Serum Calcium**

Serum total calcium, glucose, and creatinine were measured with a Hitachi 917 chemistry analyzer (Roche Diagnostics, Indianapolis, IN).

**Analysis of Tissues**

Frozen sections (7 μm) of a resected tracheal block, containing the thyroid, parathyroid glands, trachea, and surrounding muscle and soft tissue, were cut on a cryostat and stained H&E. Tissues were also fixed in formalin and embedded in paraffin. For Men1 immunostaining, tissue was excised and fixed in 4% paraformaldehyde in PBS overnight. Briefly, tissue was processed, embedded in paraffin, sectioned, and pretreated with 10 mM citric acid buffer (pH 6.0) for 7 min with boiling using a microwave. After quenching endogenous peroxidase activity with 3% hydrogen peroxide in distilled water for 10 min and blocking for 15 min with avidin D reagent (Vector Laboratories, Inc.), 15 min with biotin reagent (Vecta), and 10 min in protein blocking agent (Coulter-Immunotech) at room temperature, slides were incubated at 4°C overnight with a 1:500 dilution of purified primary rabbit polyclonal Men1 antibody. Biotinylated goat antirabbit secondary antibody (Vector Laboratories, Inc.) at a 1:200 dilution was applied for 30 min, followed by treatment with horseradish peroxidase-conjugated avidin-biotin complex reagent (Vector PK-6100). The signal was developed for up to 10 min with 3,3′-diaminobenzidine (Vector Laboratories, Inc.), and sections were counterstained with hematoxylin for 1 min. Each incubation step was followed by two 5-min PBS washes.

For Calcium Sensing Receptor immunohistochemistry, frozen sections were used. Sections were thawed and fixed in 4% paraformaldehyde in PBS overnight, and a primary rabbit polyclonal antibody (kindly provided by Dr. Dolores Shoback, University of California, San Francisco, CA) was used at a 1:200 dilution. For blocking tissue sections, primary and secondary antibodies were prepared in PBS containing 1% skim milk, 3% goat serum, 0.01% Tween 20, and 3% fish gelatin (Sigma Chemical Co.). Pathologists who were blind to the genotype of the animals then examined the slides.

**Measurement of gland size**

Serial frozen cross-sections (7 μm) of the tracheal block of the animals were reviewed by a pathologist (S. M. H.) blinded to the genotype and phenotype of the mice, and every profile containing a whole cross-section of parathyroid gland was catalogued and measured in two dimensions, at right angles with a micrometer. The largest cross-section for each individual parathyroid gland was identified (product of the two dimensions) and used in the final calculations.
PCR determination of deletion of floxed alleles

PCR was used to determine the deletion of floxed Men1 gene occurring in the parathyroid of floxed Men1 homozygous mice that were either Cre+ (group 1) or Cre− (group 4). DNA was purified from laser capture microdissected tissue from either parathyroid gland or adjacent muscle.

The test samples and the control samples were all PCR amplified using a set of three primers consisting of a forward primer, X (5′CCCACATCCAGTCCTCTCTTCAGCT3′), and two reverse primers, Y (5′ACCTACAGCCTAGCCAG3′) and Z (5′CGGAGAAAGGTAATGAAATGGC3′). A 100-ng aliquot of template DNA was used for each reaction. Primer X is located in exon 2, primer Y is located in intron 8 after the 3′loxP site and is specific for the deleted form of the gene, whereas primer Z is located upstream to the 5′lox site and is specific for the vector fragment inserted when the TSM construct was cloned (5). Therefore, the combination of primers X and Y yields a 390-bp PCR product specific for the deleted form of the gene. The nondeleted form of the gene is too long to be amplified by this PCR. In contrast, the combination of Y and Z, a 236-bp PCR product specific for the floxed gene, was used as an internal control for the PCR. PCR conditions were 95°C for 10 min; 95°C for 30 s, 60°C for 1 min, and 72°C for 3 min for 45 cycles; 72°C for 10 min. The concentrations of the PCR reagents were standard, except for the primers X, Y, and Z, which were used, respectively, in 1-, 3-, and 0.5-fold. PCR products were then run on a standard DNA gel.

Statistical Analysis

All statistical analyses were performed using a Power Macintosh G4 computer and Instat 2.01 statistical package (GraphPad Software).

RESULTS

Parathyroid-Specific Cre Expression. To direct the expression of Cre-recombinase specifically and exclusively to parathyroid tissue, we constructed a vector using the 5′upstream control region of the human PTH gene driving expression of Cre recombinase obtained from a commercially available Cre expression vector. The same human PTH promoter construct we used has been described previously (13). The PTH-Cre construct that was generated through restriction digest and ligation of the Cre recombinase expression cassette into the human PTH promoter vector is shown (Fig. 1). After microinjection and implantation of blastocysts, newborn pups were screened by PCR with primers specific for Cre using DNA isolated from tail snips (data not shown). Three PCR-positive putative founders were then screened further by Southern blot analysis (data not shown) using a radiolabeled PTH-Cre-specific sequence as a probe. The results demonstrated that integration of the PTH-Cre transgene is functional and tissue specific. PTH-Cre-positive as well as PTH-Cre-negative mice were crossed to a Z/AP reporter strain. The tracheal block was sectioned and stained by H&E as well as for alkaline phosphatase. PTH-Cre-positive mice had positive alkaline phosphatase staining in some cells of their parathyroid tissue without any evidence of alkaline phosphatase staining in any other adjacent tissues. PTH-Cre-negative mice failed to demonstrate alkaline phosphatase staining in the parathyroid gland.

Table 1 Group assignments

Mice were grouped by genotype into one of five groups. The number of mice for each group at the start of the experiment is listed under n. If the mice died or were removed for pathology, they were replaced with age- and sex-matched littermates.

<table>
<thead>
<tr>
<th>Group</th>
<th>PTH-Cre</th>
<th>Men1</th>
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<tr>
<td>1</td>
<td>+</td>
<td>dN/dN</td>
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<td>2</td>
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*a dN, Men1 flanked by loxP sites; WT, wild-type Men1.*
transgene occurred in a consistent pattern in the offspring of two of the three mice from the positive founder lines. The third mouse did not have PTH-Cre insertion confirmed by Southern blot.

The function of the PTH-Cre transgene was assayed by crossing PTH-Cre-positive mice with mice carrying the Z/AP reporter construct (11). Z/AP mice have the β-galactosidase gene flanked by loxP sites upstream from the human heat-resistant alkaline phosphatase gene. A cross between a Z/AP reporter mouse and a mouse expressing Cre-recombinase in a tissue-specific fashion will result in positive alkaline phosphatase staining in those tissues in which the gene rearrangement takes place. The activity of the expressed alkaline phosphatase will be resistant to heat inactivation. We examined the parathyroid gland in a 3-month-old PTH-Cre-positive–Z/AP-positive mouse (Fig. 2). Alkaline phosphatase staining is present in the parathyroid gland in the PTH-Cre-positive mouse (although not in all cells) but not in the parathyroid gland of the PTH-Cre-negative mouse. In addition, the surrounding tissues, including the thyroid gland, muscle, lymph node, trachea, thymus, and salivary tissues, fail to demonstrate alkaline phosphatase staining. Additional tissues were examined in these mice including lung, heart, liver, brain, stomach, spleen, kidney, large intestine, small intestine, and pancreas, and there was no alkaline phosphatase staining present in any of the tissues examined in either PTH-Cre-positive or PTH-Cre-negative mice crossed with the Z/AP reporter strain (data not shown). These results demonstrated that we had successfully constructed a transgenic mouse expressing Cre-recombinase exclusively in parathyroid tissue.

**Construction of a Transgenic Line with a Floxed Men1 Gene.**

Mice were generated that contained inserted loxP sites in introns 2 and 8 of the Men1 gene by breeding the existing line, Men1TSM+/− (5),
with EIa-Cre mice that express cre ubiquitously from the EIa promoter (10). This breeding resulted in conditionally targeted mice for Men1 that were bred to homozygosity and termed Men1ΔN/ΔN mice.

**Crosses of PTH-Cre-positive Mice with Floxed Men1 Mice.** To determine whether the tissue-specific deletion of the Men1 gene resulted in a parathyroid-specific phenotype, crosses were performed between PTH-Cre-positive mice and mice with the Men1 gene flanked by loxP sites. The progeny of these crosses were genotyped, and five groups of animals were identified and matched by age and gender. Table 1 shows the genotype of these five groups of mice with respect to presence or absence of the PTH-Cre transgene and the loxP status of the Men1 gene. These animals were followed prospectively for analysis of serum and histology.

**Measurements of Serum Calcium.** The five groups of transgenic mice were followed with serial determinations of serum calcium, glucose, and creatinine at monthly intervals from 1 month of age through 14 months of age. We measured the mean total calcium values in the blood of all groups of mice over the 14-month study (Fig. 3). By 7 months of age, serum calcium levels in group 1 mice were significantly higher than in groups 2–5. At the 9-month time point, the individual mice in group 1 had consistently higher calcium values than the mice in the other groups, and this difference was statistically significant (P < 0.0001, ANOVA). Measurements of glucose and creatinine in the serum of all five groups of mice determined at the same time as calcium values were no different between any of the groups (data not shown).

**Examination of Parathyroid Glands.** Mice were sacrificed in each of the five groups beginning at 4 months of age, and tissues were harvested including tracheal block (parathyroid, thyroid, muscle, esophagus, trachea, and salivary glands) brain, heart, lung, liver, kidney, spleen, pancreas, stomach, small intestine, and large intestine. Tissues were frozen as well as formalin fixed, sectioned, and stained by H&E and immunostaining. No abnormalities were found in any tissues other than the parathyroid glands. We compared histological sections of parathyroid glands in mice from group 1 to parathyroid glands from mice in group 4 (Fig. 4). Histological examination of the group 1 mice at 9 months of age showed larger parathyroid glands with increased numbers of densely packed and disorganized follicular cells. The group 1 mice had parathyroids that had increased in size sufficient to no longer be neatly embedded into the thyroid gland but rather pushed into the adjacent skeletal muscle. Such changes were seen in over 80% of the group 1 mice examined. Abnormalities were not seen in any of the other tissues examined. The gland sizes are determined and are shown in Fig. 5. Gland size determinations were made on 14-month-old mice and demonstrated that the mean group 1 gland size for five representative hypercalcemic mice was significantly larger than five representative normocalcemic group 4 mice (group 1 mean = 907 mm² ± 278.4 mm² SE versus group 4 mean = 188.4 mm² ± 29 mm² SE; P = 0.016, Mann-Whitney U test).

**Immunohistochemistry** was performed for menin expression and the expression of the calcium-sensing receptor. Fig. 6, A–D, shows loss of staining for menin in group 1 mouse parathyroids compared with age-matched group 4 controls. There is also evidence of decreased expression of the calcium-sensing receptor in lesions found in group 1 mice (Fig. 6, E and F).

**Laser-capture microdissection** was performed on the parathyroid gland and adjacent muscle in both group 1 mice as well as group 4 mice. DNA was purified from these cells and subjected to PCR using primers that would indicate the presence of a loxP site as a determination of the presence of DNA as well as primers that would determine whether or not deletion had taken place in the target tissue. For the parathyroid glands in group 1 mice, deletion of the Men1 gene in

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**DISCUSSION**

Hypercalcemia can be a profoundly debilitating condition in both nonfamilial and familial parathyroid neoplasia (14). For patients with MEN1, uncontrolled parathyroid neoplasia and the resulting hypercalcemia frequently lead to urinary calculi and can cause bone demineralization and the acceleration of osteoporosis. A better understanding of the pathophysiology of this condition would undoubtedly help investigators develop new strategies for the treatment of this condition. A more thorough understanding of the pathophysiological consequences of a MEN1 deletion in parathyroid tissue would not only be relevant to the study of the MEN1 syndrome but also to the study of primary hyperparathyroidism, in general. As many as 30% of sporadic cases of primary hyperparathyroidism can be shown to have a somatic deletion of MEN1 in parathyroid tissue (15).

We described previously a conventional knockout model for MEN1 that resulted in pathological parathyroid neoplasia, but hypercalcemia was not observed (5). Here, we describe a new model that allows for the study of mice with a homozygous deletion of the Men1 tumor suppressor gene specifically in the parathyroid glands, thus allowing for live births and the ability to study the consequence of a Men1 gene deletion in the tissue of interest. These mice develop hypercalcemia as early as 7 months of age. These mice also have pathological features consistent with parathyroid neoplasia and are spared MEN1-related neoplasms in any other tissues.

Allelic deletion of Men1 in the parathyroids of Cre-expressing mice homozygous for the floxed Men1 allele was demonstrated using PCR. Adjacent muscle tissue in these mice failed to demonstrate any evidence of deletion. Mice homozygous for floxed Men1 that were not expressing Cre had no evidence of deletion in parathyroid or muscle. These mice also had abnormal parathyroid glands by histological examination, with increased gland size, increased numbers of cells, and loss of the normal organization of the gland. These mice had significantly elevated serum calcium levels. These pathological and clinical findings are similar to those seen in patients with MEN1 hyperparathyroidism. In fact, the pathological appearance of the ab-
normal glands in the mice is very similar to the appearance of abnormal glands in humans. Although mice have two parathyroid glands (compared with the typical four in humans), both glands were found to be pathologically abnormal in the group 1 mice. This evidence of multigland disease is comparable with the human condition.

Furthermore, immunostaining confirmed lower levels of expression of the menin protein in group 1 mice versus group 4 controls. Immunostaining also showed reduced expression of the calcium-sensing receptor in neoplastic parathyroids from mice in group 1. Reduced calcium-sensing receptor expression has been reported in primary and uremic secondary hyperparathyroidism in humans, but not to our knowledge in MEN1 (16). Group 1 mice also demonstrated significantly larger parathyroid glands by 14 months of age compared with age- and gender-matched controls. The documented parathyroid specificity of Cre recombinase expression in the PTH/Cre transgenic mice we generated suggests that these mice should prove useful in generating mouse models of parathyroid-specific deletion of other genes of interest, such as the extracellular calcium-sensing receptor and the vitamin D receptor.

The mouse model of primary hyperparathyroidism we created will allow the study of a variety of questions of pathophysiological and therapeutic interest in an in vivo system. These mice should be useful in testing candidates for treatment of hypercalcemia as well as for preventing or reversing parathyroid neoplasia. Identification of promising targets for such treatments will require detailed studies of the mechanistic basis for parathyroid neoplasia subsequent to loss of menin function and elucidation of the linkage between parathyroid neoplasia and abnormal calcium regulation. For example, there are likely other genetic and epigenetic consequences in the parathyroid resulting from the loss of the Men1 tumor suppressor gene. These mice may allow for a better elucidation of the pathways responsible...
for parathyroid neoplasia by direct molecular genetic studies of the parathyroid tumors arising in these mice and by crosses of these mice with other genetically altered mouse strains. Crosses of these mice with other transgenic models of parathyroid neoplasia and hypercalciemia, such as mice that overexpress cyclin D1 in the parathyroid glands (13), may allow us to better understand the complex pathways involved in calcium homeostasis.

In conclusion, we have successfully developed a mouse model of primary hyperparathyroidism caused by parathyroid-specific deletion of the Men1 gene. Using a targeted tissue-specific knockout strategy, we have demonstrated that the deletion of Men1 results in histological findings consistent with parathyroid neoplasia and serum hypercalcemia by 7 months of age. It is our hope that this model will serve as a useful tool for the study of hyperparathyroidism and for the molecular basis of neoplasia caused by loss of menin function.

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Parathyroid Gland-specific Deletion of the Mouse Men1 Gene Results in Parathyroid Neoplasia and Hypercalcemic Hyperparathyroidism

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