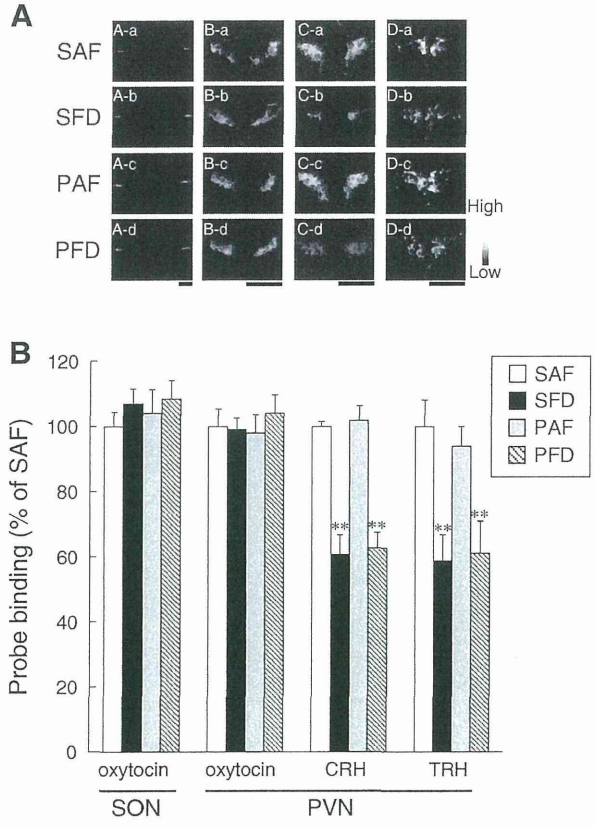

Fig. 5 Feeding-regulating peptides in the SON and the PVN **a** Digital images of ISH of *oxytocin* in the SON (*A-a–A-d*) and the PVN (*B-a–B-d*), *CRH* in the PVN (*C-a–C-d*), *TRH* in the PVN (*D-a–D-d*). *Scale bar* 1 mm. **b** Quantification of mRNA signals using MCID. PAF data are presented as 100 %. Data are presented as mean ± SEM. $^{**}P < 0.01$ vs. SAF and PAF

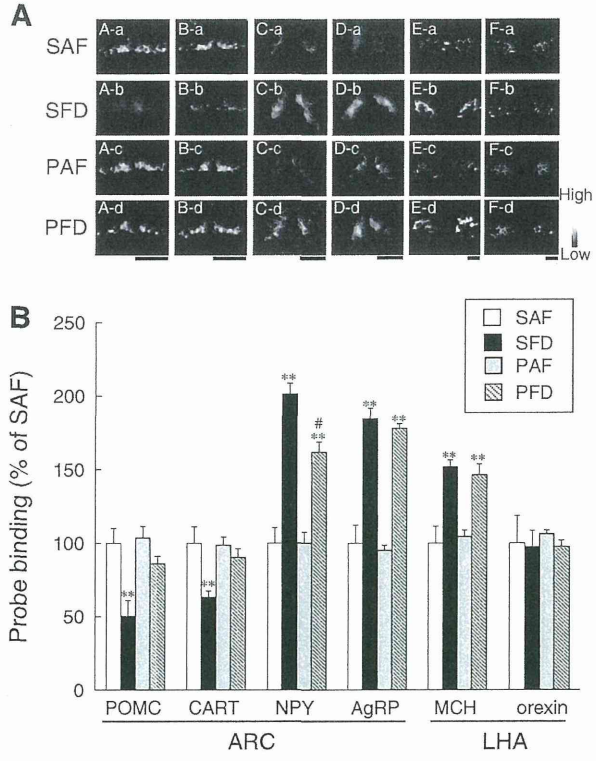

Fig. 6 Feeding-regulating peptides in the ARC and the LHA **a** Digital images of ISH of *POMC* in the ARC (*A-a–A-d*), *CART* in the ARC (*B-a–B-d*), *NPY* in the ARC (*C-a–C-d*), *AgRP* in the ARC (*D-a–D-d*), *MCH* in the LHA (*E-a–E-d*), and *orexin* in the LHA (*F-a–F-d*). *Scale bar* 1 mm. **b** Quantification of mRNA signals using MCID. PAF data are presented as 100 %. Data are presented as mean ± SEM. $^{\#}P < 0.05$ vs. SFD, $^{**}P < 0.01$ vs. SAF and PAF

After PCPA systemic treatment, the body weight decreased in PFD and PAF (Fig. 2) compared to SAF. Although the body weight in PAF after day 3 decreased significantly compared to SAF, food intakes after day 3 were comparable to SAF; thus, it was expected that there would be no significant effects on the gene expression of the feeding-regulating neuropeptides in the hypothalamus in our experimental situation after day 3.

Cumulative food intake in PFD decreased at days 4, 5, and 6 compared to those in SFD (Fig. 3). After food deprivation in the PCPA-treated group, the gene expression of the *POMC*, *CART*, and *NPY* seemed likely to be involved in 5-HT system in the hypothalamus according to our results of ISH which will be described later. Although this cumulative food intake data appear to suggest that serotonin facilitates food intake which possibly contradicts the previous concept that serotonin inhibits food intake, the body weights themselves also decreased after serotonin depletion. The decrease of body weights may be caused by complex factors, not just by decreased food intake. Thus, we decided to evaluate accumulating food intake but not food intake per body weight. This analysis appears on the surface to produce opposite results when the food intake is expressed as per body weight, but in fact complex factors may be involved in decreased food intake in each animal after 5-HT-depletion. This point should be examined by further study. We have not examined the gene expression of the feeding-regulating peptides in the hypothalamus after re-feeding; however, it is speculated that some of the feeding-regulating peptides in the hypothalamus may not change despite re-feeding with PCPA treatment.

The significant decrease in PG in SFD and PFD compared to that in SAF and PAF might have been due to the effect of food deprivation. No statistical differences were seen between SAF and PAF or between SFD and PFD, and P-Osm did not differ among all the experimental groups, suggesting that we can compare the feeding-regulating neuropeptides in the hypothalamus in this experiment. Plasma 5-HT dramatically decreased after PCPA treatment (Fig. 4a), but not as completely as that of the hypothalamus. And it tended to decrease with food deprivation.

These result suggests that peripheral 5-HT was also degraded by PCPA as was hypothalamic 5-HT. As previously described [23, 24], food restriction decreased 5-HT levels, which is consistent with our results. Because leptin is a satiety molecule [25, 26], it significantly decreased in food deprivation. But there were no statistical changes between SAF and PAF or SFD and PFD, suggesting that PCPA treatment did not affect plasma leptin levels.

Food deprivation for 48 h significantly increased plasma desacyl ghrelin concentration, indicating the adaptive response to starvation [27]. This was the case in plasma-active ghrelin in SFD; however, it significantly increased in PAF and PFD. These results may indicate that PCPA systemic treatment itself decreased food intake, which resulted in increasing active ghrelin secretion as a secondary response. One possible explanation is that a large amount of active ghrelin had already been secreted by the PCPA treatment in PAF and additional food deprivation produced no further changes in PFD. However, it is difficult to evaluate whether 5-HT may be involved in the regulation of circulating active ghrelin from the data obtained from the present study. Further study should be carried out to confirm this and to elucidate its mechanism.

Among all the feeding-regulating neuropeptides we examined, there was no remarkable difference between SAF and PAF, which indicates that PCPA itself did not affect the peptides under the free feeding condition. Therefore, if the feeding-regulating peptide was markedly different between SFD and PFD, it may be said that the peptide is regulated via the serotonergic system in the hypothalamus.

The gene expression of the *oxytocin*, which is recognized as an anorexic peptide [28], in the SON and the PVN, did not differ despite food deprivation (Fig. 5a, b). It is considered that no significant difference can be detected as a state of *oxytocin* mRNA. Further study, such as a comparison with heteronuclear RNA expression [29], is needed.

The gene expression of the *CRH* and the *TRH* in the PVN decreased significantly after 48 h food deprivation (Fig. 5a, b), as previously described [9, 11–13]. They also decreased after food deprivation under the PCPA pretreatment, which suggests that 5-HT in the brain does not affect the gene expression of *CRH* and *TRH*. Although it has been described that 5-HT 2C receptors exists in the CRH neuron and that there is a possible interaction between 5-HT and CRH [30], our experiment was performed under a condition of almost completely depleted 5-HT, and we examined only one point, so a simple comparison cannot be made. The time course of the gene expression of the *CRH* may have gradually changed after the start of food deprivation.

As expected, the gene expression of the *POMC* and *CART* decreased significantly after food deprivation (Fig. 6a, b). Strikingly, however, they remained unchanged by PCPA pretreatment despite food deprivation. Thus, the effects of PCPA are not statistically confirmed. CART in the ARC is co-stored with POMC [31] and 5-HT receptors exist in the POMC neuron in the ARC [17, 32]. Our results indicate that the serotonergic system in the hypothalamus may project to the POMC and CART neurons in the ARC, which is consistent with previous reports [17, 33].

The gene expression of the *NPY* in the ARC increased significantly after food deprivation (Fig. 6a, b). While the gene expression of the *NPY* also increased significantly after food deprivation with PCPA pretreatment, the extent of the rate of increase was not uniform. The gene expression of the *NPY* in PFD did not increase adequately compared to SFD. As the NPY neurons in the ARC are also expressed in 5-HT receptors [34], there may have been a possible interaction with 5-HT. Taken together with our results, 5-HT may be partially related to NPY regulation.

As previously reported [15], the gene expression of the *MCH* increased significantly after food deprivation (Fig. 6a, b). The variation was to the same degree as with PCPA pretreatment. Our results indicate that 5-HT had no effect to the gene expression of the *MCH*. As with the result of *oxytocin*, the gene expression of the *orexin* did not differ despite food deprivation, which indicates that 5-HT in the hypothalamus may not affect the gene expression of the *MCH*.

In conclusion, we have shown that there may have been possible interaction between the serotonergic system in the hypothalamus and the gene expression of the *POMC*, *CART*, and *NPY* in the ARC. These findings provide evidence of the mechanism of the neural circuit that mediates feeding-regulating neuropeptide reaction in response to 5-HT in the hypothalamus.

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Conflict of interest All authors declare that they have no conflict of interest.

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NOTE

Caspase 8 and menin expressions are not correlated in human parathyroid tumors

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Abstract. Menin is lost by the sequential inactivation of both *MEN1* alleles in subsets of non-hereditary endocrine tumors as well as those associated with multiple endocrine neoplasia type 1 (MEN1), an autosomal dominant hereditary cancer syndrome characterized by multiple tumors including parathyroid, pituitary and enteropancreatic endocrine tumors. Loss of menin has been reported to be associated with lowered caspase 8 expression and resistance to apoptosis in murine fibroblasts and in pancreatic islet tumors arising in heterozygous *MEN1* gene knockout mice, the animal model of the human MEN1 syndrome. We confirmed by menin-knockdown experiments with specific siRNA that menin is crucial for caspase 8 expression in human culture cells while overexpression of menin did not increase caspase 8 protein over basal levels. We then examined expression of menin, caspase 8 and cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p15^{Ink4b}$ by Western blotting in human parathyroid tumors surgically resected from patients with MEN1 and those with non-hereditary primary hyperparathyroidism. The menin and $p27^{Kip1}$ expression levels were correlated with *MEN1* mutation status that was confirmed by DNA analysis. The caspase 8 and $p15^{Ink4b}$ protein levels were variable among tumors, and were not correlated with menin protein levels. These findings suggest that human endocrine tumors lacking menin may not always exhibit lowered caspase 8 expression and hence may not be resistant to apoptosis-inducing therapy.

Key words: MEN1, Menin, Caspase 8, siRNA

MENIN is the protein product of the tumor suppressor gene *MEN1*, the causative gene of multiple endocrine neoplasia type 1 (MEN1), an autosomal dominant familial cancer syndrome characterized by the multiple occurrence of endocrine tumors, such as pituitary, parathyroid and pancreatic islet tumors [1, 2]. Following the inactivation of the both *MEN1* alleles, the wild-type menin is lost in the tumors associated with MEN1. Menin is also frequently lost in some non-hereditary, sporadic endocrine tumors by somatic mutations of the both *MEN1* alleles. For example, 21 % and 39 % of sporadic parathyroid tumors have been reported to exhibit somatic *MEN1* gene mutation and loss of heterozygosity (LOH) of the *MEN1* locus, respectively [3]. These findings suggest that the complete loss of menin is an important step in the development of not only hereditary but also subsets of sporadic endocrine tumors.

Menin is ubiquitously expressed and mainly targeted to the nucleus [4, 5]. Although its molecular function is not fully understood, menin has been shown to modify chromatin structure and thereby regulate the transcription of many target genes including cyclin-dependent kinase inhibitor (CDKI) genes $p18^{Ink4c}$ and $p27^{Kip1}$ [2, 6]. Menin-induced expression of these CDKI genes seems to be relevant to tumor suppressor function of menin because inactivating germline mutations of the CDKI genes $p15^{Ink4b}$, $p18^{Ink4c}$, $p21^{Cip1}$ and $p27^{Kip1}$ are known to cause MEN1-like syndromes [7, 8].

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On the other hand, previous studies have shown that menin plays a crucial role in promoting apoptosis, which depends on proapoptotic proteins of Bax and Bak [9]. Menin has also been demonstrated to induce the expression of caspase 8 mRNA and protein by enhancing histone acetylation at the 5'-untranslated region of the caspase 8 gene in murine embryonic fibroblasts [10]. Moreover, caspase 8 expression has been shown to decline in pancreatic islets and insulinomas in heterozygous *MEN1* gene knockout mice, the murine model of the human MEN1 syndrome [10]. These findings suggest that menin-dependent caspase 8 expression plays a role in tumor suppression. Because caspase 8 is a key protein in the death-receptor pathways, tumors lacking caspase 8 may be resistant to apoptosis-inducing therapy [11].

Although the endocrine tumors in the murine MEN1 model showed decreased caspase 8 expression, it remains uncertain whether the human endocrine tumors lacking menin exhibit similarly lowered caspase 8 levels. To clarify the relationships between caspase 8 and menin expression levels in human tumors, we have studied caspase 8 proteins in human culture cells and in parathyroid tumors associated with MEN1 and non-hereditary parathyroid tumors. We report here that caspase 8 expression was down-regulated by menin knockdown in human culture cells, but human parathyroid tumors had variable amounts of caspase 8 proteins irrespective of menin expression levels.

Materials and Methods

Cell culture and transfection of expression plasmid and siRNA

WI38-VA13 and GM0639 cells, the human fibroblasts derived from normal persons and immortalized with SV40, and HEK293T cells, the transformed human embryonic kidney cells, were maintained in DMEM supplemented with 10 % fetal bovine serum and antibiotics as described previously [12]. GM0639 cells were treated with 50 μM etoposide for 48 h to be served as a control for procaspase and cleaved caspase proteins in Western blotting.

The menin-expressing plasmid was constructed by inserting the protein-coding region of the menin cDNA plasmid (p1533, kindly donated by Dr. M. Ohki and Dr. F. Hosoda, National Cancer Center Research Institute, Japan) into the unique *Hind*III site of the pRc/CMV expression vector (Invitrogen, USA) after blunting each end as described previously [5] . The plasmid was transfected with FuGENE6 transfection reagent (Roche, USA) and were harvested 48 h after transfection.

Menin siRNA or scrambled negative control siRNA (menin siRNA (h), sc-35922 and control siRNA-A, sc-37007, Santa Cruz Biotechnology, USA) were transfected by the procedures recommended by the supplier into culture cells that were seeded at 5×10^5 cells per 35 mm plate the day before transfection. After transfection, cells were cultured in normal growth media for 48 h before collection.

Real-time RT-PCR

Total RNA was extracted from cells with the RNeasy Mini Kit (Qiagen, USA). Specific mRNA was quantified by real-time PCR using a Fluorescent Quantitative Detection system (BioFlux, USA) with QuantiTect SYBR Green RT-PCR assay kits (Qiagen). The mRNA value for each gene was normalized relative to the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in RNA samples. The following PCR primers were used for each mRNA. Menin: sense, TTAGGGAACCTGGCAGATCTAGAG; antisense, GTAGCCAGCCAGGTACATGTAGG; GAPDH: sense, TGCACCACCAACTGCTTAGC; antisense, AGTGATGGCATGGACTGTGG. Statistical comparison of mean values was performed by the Student's *t*-test. Differences with a p value of less than 0.05 were considered statistically significant.

Western blotting

Whole cell lysates were prepared as described previously [12]. Protein samples separated by electrophoresis and blotted onto nitrocellulose membrane (Invitrogen) were subjected to antibody binding as described previously [13]. The following antibodies were used: goat polyclonal anti-menin (C-19, sc-8200), rabbit polyclonal anti-$p27^{Kip1}$ (C-19, sc-528), rabbit polyclonal ant-$p15^{Ink4b}$ (K-18, sc-613), anti-mouse IgG-HRP and anti-goat IgG-HRP antibodies were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-actin antibody (clone C4, #MAB1501) was obtained from Millipore (USA). Mouse monoclonal anti-caspase 8 antibody (12F5, ALX-804-242) was provided by ENZO Life Sciences (USA). The blots were visualized by

Table 1 Parathyroid tumors examined.

Parathyroid Tumor	Germline *MEN1* mutation#	Predicted effect of germline mutation	LOH	Clinical features
1	c.1628C>G	S543X (termination at codon 543)	+	Recurrent parathyroid tumors
2*	c.893delC	frame shift at codon 298	+	Multiple parathyroid tumors, Gastrinoma; Familial
3	c.1072G>T	E358X (termination at codon 358)	ND	Multiple parathyroid tumors, Glucagonoma, Prolactinoma; Familial
4a, 4b	c.492delC	frame shift at codon 164	+	Recurrent parathyroid tumors, Acromegaly
5	c.1350+1_1350+14 del14insCT	abnormal splicing at the 3' end of exon 9	+	Familial hyperparathyroidism
6*	large deletion	gene deletion	+	Multiple parathyroid tumors, Gastrinoma, Adrenocortical tumor; Familial
7-12	none	-	ND	Sporadic parathyroid tumor

Tumors 4a and 4b are from different parathyroid glands of a single patient.
#, Mutations are designated by the standard nomenclature [19]. *, reported previously [20, 21]. ND, not determined.

enhanced chemiluminescence method (RPN2135, GE Healthcare, UK) and imaging system LAS3000 (Fujifilm, Japan). Images were analyzed with the ImageJ software.

Blood and tumor specimens

Patients with primary hyperparathyroidism were diagnosed as having non-hereditary, sporadic parathyroid tumor or hereditary tumors in the local hospitals (Table 1). The diagnosis of MEN1 was confirmed by DNA analysis for heterozygous germline *MEN1* gene mutation. Seven parathyroid tumors surgically removed from 6 patients affected with MEN1, including 2 tumors arising from different parathyroid glands of a patient (case 4), and 6 parathyroid tumors from 6 patients with non-hereditary, sporadic parathyroid tumor were examined. The tumors were pathologically diagnosed as benign multiple adenomas or hyperplasia in the MEN1 cases and as benign adenoma in sporadic cases.

Peripheral blood was collected from all MEN1 patients except for one patient (case 3) and used for the confirmation of germline mutation and LOH analysis. Prior informed consent was obtained for germline mutation analysis of blood DNA. Some tumors were analyzed after unlinkable anonymization without informed consent for gene analysis. This study was approved by the ethics committee for gene analysis research of the National Cancer Center and Kobe University Graduate School of Medicine.

Analysis of MEN1 gene mutation and loss of heterozygosity

The *MEN1* gene sequence in the blood and tissue DNA was analyzed for mutations by the direct nucleotide sequencing of PCR-amplified exons as described previously [14]. In a MEN1 patient whose germline mutation was not detectable by the conventional sequencing method (case 6), a gross deletion involving exon 8 was detected by PCR-based DNA walking (manuscript in preparation). Loss of heterozygosity in the chromosome 11q13 region was examined at the polymorphic DNA marker sites, *PYGM*, *D11S4940* and *D11S4946*, as described previously [15]. In addition, reduction of the wild type sequence in the tumor DNA at the site of heterozygous germline mutation was also considered as LOH.

Results

Effects of knockdown and overexpression of menin on caspase 8 expression in human cell lines

Because menin knockdown has been shown to suppress caspase 8 expression in murine cells, we examined the effect of menin on caspase 8 expression in human cell lines derived from normal fibroblasts.

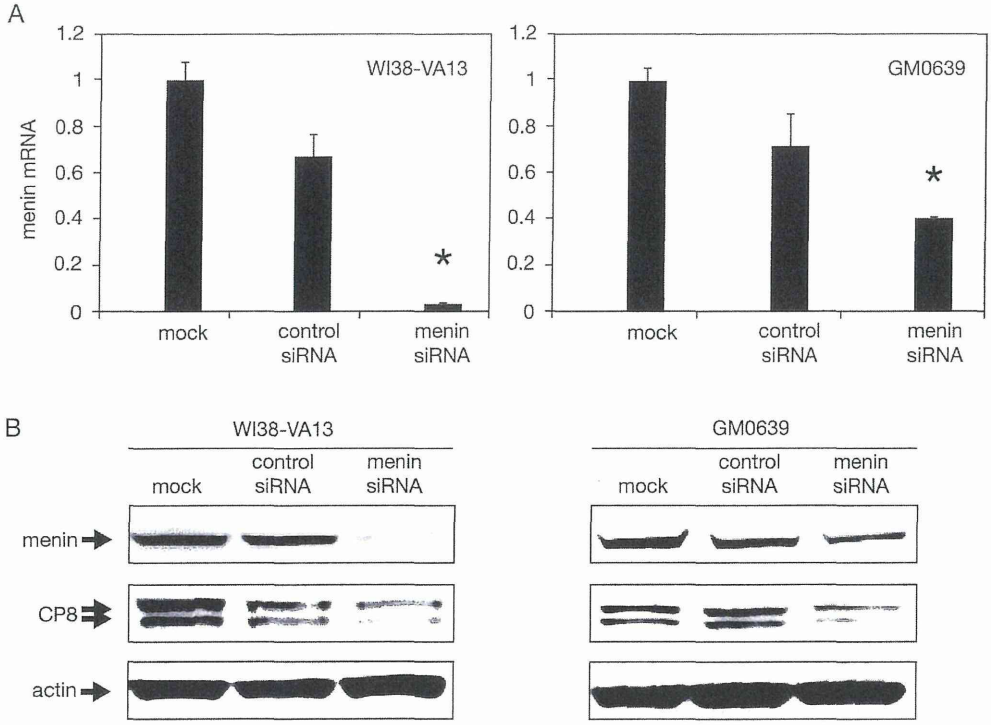

Fig. 1 Caspase 8 expression in menin knockdown experiments. Cells were transfected with control siRNA or menin siRNA or treated with transfection reagents without RNA (mock). (A) The amounts of menin mRNA measured by quantitative RT-PCR in WI38-VA13 (left) and GM0639 (right) cells. Data are presented as the relative value to the mean value of mock-transfected cells and the standard error from three experiments. *, $p<0.05$ versus cells transfected with control siRNA (n=3). (B) Representative Western blot of menin and caspase 8 (CP8) in WI38-VA13 (left) and GM0639 (right) cells. Actin was served as a loading control.

Introduction of menin siRNA into WI38-VA13 and GM0639 cells reduced the menin mRNA and protein levels (Fig. 1). The caspase 8 expression levels were also decreased in the both cell lines by the transfection of menin siRNA (Fig. 1). On the other hand, menin overexperssion in WI38-VA13, GM0639 and HEK293T cells by the transfection of menin expression plasmid did not change procaspase 8 protein levels (Fig. 2). These findings suggest that menin is crucial for caspase 8 expression and that these cells contain sufficient amount of endogenous menin which allow full expression of procaspase 8 protein.

MEN1 gene status in parathyroid tumors

In order to examine the effect of menin in human tumors, parathyroid tumors surgically removed from patients affected with MEN1 were used as tumors lacking menin (Table 1). The blood DNA analysis confirmed the presence of germline mutations in all the MEN1 patients except for case 3 whose blood was not available for this study. Comparison of blood and tumor DNA sequences and polymorphic markers revealed LOH in all tumors from the MEN1 patients except for case 3. In the tumor DNA of case 3, which showed the typical manifestations of MEN1 with positive family history of endocrine tumors, the mutated sequence TAG at codon 358 was predominant and the wild type sequence GAG was almost absent (data not shown). These findings suggest that the nonsense mutation E358X (GAG>TAG) is the germline mutation of this family and that the wild-type allele was lost in the tumor cells although it is theoretically possible that a large genomic deletion is the germline mutation of this family and that E358X is a somatic mutation. Altogether, the absence of the normal *MEN1* gene was confirmed in the neoplastic cells of all the tumors from the MEN1 patients examined.

Sporadic parathyroid tumors were used as counterpart specimens, which had normal *MEN1* alleles (Table 1). Because *MEN1* gene inactivation is also

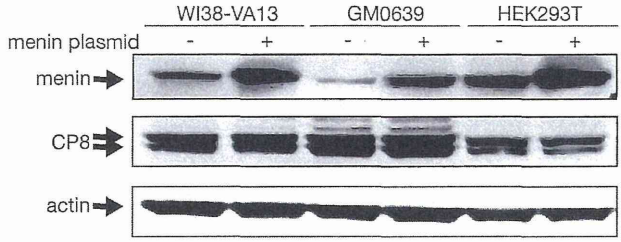

Fig. 2 Menin and caspase 8 (CP8) expression detected by Western blotting in WI38-VA13, GM0639 and HEK293T cells transfected with menin-expressing plasmid (+) or vehicle (-). Actin was served as a loading control.

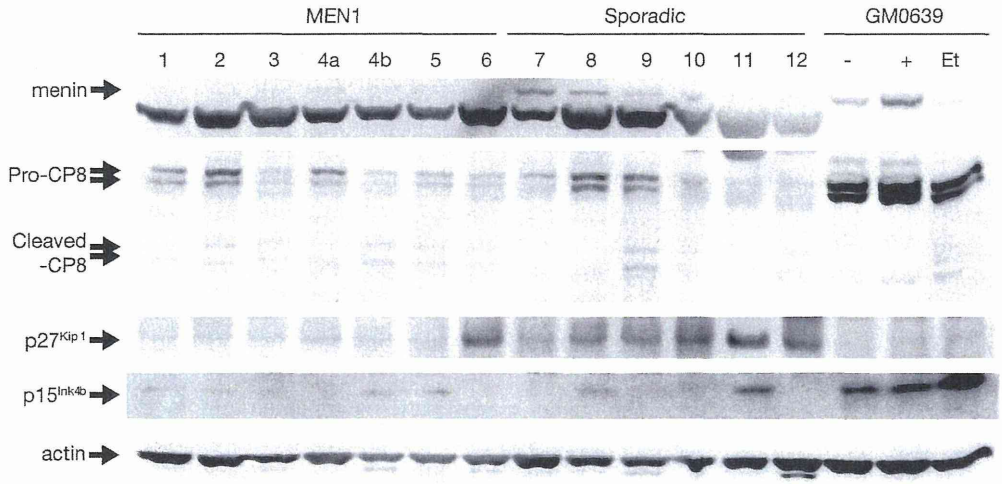

Fig. 3 Menin, caspase 8, $p27^{Kip1}$ and $p15^{Ink4b}$ expression detected by Western blotting in human parathyroid tumors. The samples in lanes 1 - 6 and lanes 7 – 12 are tumors from the MEN1 patients and sporadic tumors, respectively, and correspond to those summarized in Table 1. GM0639 cells transfected with menin-expressing plasmid (+) along with non-transfected cells (-) were used as the size marker for normal menin. GM0639 cells treated with etoposide (Et) were used as the size marker for procaspase 8 (Pro-CP8) and cleaved caspase 8 (Cleaved-CP8). Actin was served as a loading control.

involved in a subset of sporadic parathyroid tumors, we selected only the tumors that had no detectable *MEN1* gene mutation. In order to minimize the possibility of including tumors having *MEN1* gene mutation that escape detection in our mutation analysis, we further selected sporadic tumors that exhibited at least one heterozygous normal polymorphic sequences at codon 418 (GAC/GAT) or codon 541 (GCA/ACA) [1], which are usually hemizygous in tumors involving *MEN1* gene inactivation.

Menin, caspase 8 and CDKI protein levels in parathyroid tumors

Western blotting analyses confirmed that menin protein levels were markedly reduced in parathyroid tumors from MEN1 patients compared with sporadic tumors although some variability was observed (Fig. 3). The faint band of menin with the authentic size detected in the MEN1 tumors should represent menin protein in the non-neoplastic cells in the tumor tissue.

Procaspase 8 protein levels were much more variable among tumors, and there was no constant correlation between menin and procaspase 8 levels (Fig. 3). The cleaved caspase 8 also showed variable expression levels. Even the individual tumors from the same patient (4a and 4b) showed different caspase 8 expression levels. These findings suggest that, while reduction of menin causes decreased procaspase 8 expression in human culture cells *in vitro*, the procaspase 8 and cleaved caspase 8 protein levels in human parathyroid tumors are not simply determined by menin

expression levels but also regulated by other unknown mechanisms.

The expression levels of CDKIs $p27^{Kip1}$ and $p15^{Ink4b}$ were also examined (Fig. 3). The amount of $p27^{Kip1}$ was reduced in all tumors from MEN1 patients except for one case (case 6) while only one case (case 7) of sporadic tumor showed reduced $p27^{Kip1}$ expression. Thus, the expression of $p27^{Kip1}$ was roughly correlated with menin expression levels. On the other hand, the expression of $p15^{Ink4b}$ was not related to *MEN1* gene mutation status. We also attempted to compare $p18^{Ink4c}$ and $p21^{Cip1}$ expression levels in these tumors but failed to detect reproducible specific signals.

Discussion

Menin has been reported to up-regulate the expression of caspase 8 by activating gene transcription and enhance apoptosis in murine fibroblasts [9]. It has also been demonstrated that caspase 8 expression is reduced in the pancreatic islet tumors occurring in the heterozygous *MEN1* gene knockout mice, the murine model of the human MEN1 syndrome [10]. However, caspase 8 expression has not been examined in the human endocrine tumors, in which the *MEN1* gene is completely inactivated. We compared caspase 8 and menin expression levels in human parathyroid tumors from patients affected with MEN1 and non-hereditary, sporadic hyperparathyroidism, and found that caspase 8 protein levels were not correlated with menin protein levels in these tumors.

The discrepancy is not probably due to the species difference because our *in vitro* experiments employing human cells reproduced menin-dependent caspase 8 expression similar to the previous findings with the murine cells. It is also unlikely that an aberrant caspase 8 gene expression is simply associated with oncogenic transformation of endocrine cells because caspase 8 levels have been shown to decline in the murine pancreatic islet tumors lacking menin [10], although the difference of the cell lineage might explain the discrepancy. Examination of human pancreatic islet tumors with verified *MEN1* gene mutations may clarify this possibility.

Another possibility of the apparent discrepancy is the difference of the tumor environments. It is suggested that the basal caspase 8 expression levels are controlled through signals culminating in the activation of SP1 and ETS-like transcription factors [11]. Interferon gamma and retinoic acids have been reported to induce caspase 8 expression in some tumor cell lines [11]. Such extracellular signals may be involved in the regulation of parathyroid cell growth and apoptosis under the physiological condition, and leading to variable caspase 8 expression levels. The distinct caspase 8 expression patterns in the individual tumors form the same patient (4a and 4b) (Fig. 3) may reflect the difference of tumor microenvironment. Alternatively, genetic or epigenetic change of the caspase 8 gene might explain the variable expression. Caspase 8 gene has been shown to be deleted in neuroblastoma [16]. In addition, caspase 8 gene is frequently inactivated by silencing through DNA methylation in various tumors including neuroblastoma and lung carcinoma [11, 16]. The human parathyroid tumors are usually diagnosed and removed after long periods of tumor growth, while tumors developed in the knockout mice were examined earlier, usually less than a year after birth. Therefore, alterations of the caspase 8 gene *per se* might be more prevalent in human tumors than in the murine models. Our present study revealed complex caspase 8 expression patterns in human tumors that may not simply reflect the phenomenon observed under experimental conditions.

In contrast to caspase 8 expression, $p27^{Kip1}$, which was also demonstrated to be up-regulated by menin at the transcriptional level [6], was more abundant in sporadic parathyroid tumors than in tumors from MEN1 patients. These findings are consistent with those reported previously [6] and support the important role of menin in regulating $p27^{Kip1}$ expression. Interestingly, we found no correlation of $p15^{Ink4b}$ protein levels with *MEN1* gene mutation status. The expression of $p15^{Ink4b}$ has also been shown to be up-regulated by menin [17]. However, the previous chromatin immunoprecipitation studies suggested that the enhancing effect of menin on $p15^{Ink4b}$ gene expression may be indirect in contrast to that on $p27^{Kip1}$ gene, which is likely to be regulated through direct binding of menin-containing protein complex to the gene promoter region [17]. Our findings are in line with the previous report that $p15^{Ink4b}$ mRNA levels were not correlated with *MEN1* gene mutation status in human pancreatic endocrine tumors [18]. The $p15^{Ink4b}$ gene promoter hypermethylation has also been demonstrated to be associated with gene silencing in some endocrine tumors [18].

In conclusion, we observed coordinated expression of $p27^{Kip1}$ and menin in human parathyroid tumors but variable expression levels of caspase 8 and $p15^{Ink4b}$ irrespectively of *MEN1* gene mutation status. Because caspase 8 is a crucial component in the apoptosis pathway, low caspase 8 expression is implicated in the resistance against apoptosis-inducing therapy of tumors [11] . Our findings suggest that the human endocrine tumors lacking menin may not always exhibit lower caspase 8 protein expression and hence may not be resistant to such therapy.

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