Molecular and Cellular Endocrinology 313 (2009) 1–8

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Regulation of vitamin D receptor function in MEN1-related parathyroid adenomas

Koen M.A. Dreijerink[a,b], Radhika A. Varier[a], Rick van Nuland[a], Roel Broekhuizen[c], Gerlof D. Valk[b], Jacqueline E. van der Wal[d], Cornelis J.M. Lips[b], J. Alain Kummer[c,1], H.Th. Marc Timmers[a,*]

[a] Department of Physiological Chemistry, University Medical Center Utrecht, Utrecht, The Netherlands
[b] Department of Internal Medicine, University Medical Center Utrecht, Utrecht, The Netherlands
[c] Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands
[d] Department of Pathology, University Medical Center Groningen, Groningen, The Netherlands

ARTICLE INFO

Article history:
Received 15 March 2009
Received in revised form 24 July 2009
Accepted 26 August 2009

Keywords:
Multiple endocrine neoplasia type 1
Menin
Parathyroid adenoma
Vitamin D receptor
Histone methyltransferase
Gene transcription
Histone modification

ABSTRACT

Multiple endocrine neoplasia type 1 (MEN1) is a heriditary syndrome characterised by the occurrence of parathyroid, gastroenteropancreatic and pituitary tumours. The *MEN1* gene product, menin, co-activates gene transcription by recruiting histone methyltransferases for lysine 4 of histone H3 (H3K4). We investigated whether in MEN1 tumours global changes in H3K4 trimethylation (H3K4me3) occur or whether alterations in gene expression can be observed. By immunohistochemistry we found that global levels of H3K4me3 are not affected in MEN1-related parathyroid adenomas. Menin can interact directly with the vitamin D receptor (VDR) and enhance the transcriptional activity of VDR. Messenger RNA levels of VDR target genes *CYP24* and *KLK6* were significantly lower in MEN1 parathyroid adenomas compared to normal tissue. Thus, aberrant gene expression in MEN1 tumours is not caused by lower global H3K4me3, but rather by specific effects on genes that are regulated by menin-interacting proteins, such as VDR.

1. Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an inherited syndrome that is characterised by the occurrence of tumours of the parathyroid glands, the pancreas and duodenum, the pituitary gland, the adrenal glands as well as neuro-endocrine tumours of the thymus, lungs and stomach, often at a young age (Brandi et al., 2001). MEN1 is caused by germ line mutations in the *MEN1* gene (Chandrasekharappa et al., 1997). To date, more than 450 different germ line mutations have been identified, most of which are clearly inactivating (Lemos and Thakker, 2008). The *MEN1* gene is a tumour suppressor gene: loss of the wild type allele is required for a cell to become a tumour cell (Larsson et al., 1988). The *MEN1* gene is expressed ubiquitously and encodes the protein menin. Menin is localised in the nucleus and can take part in many cellular processes such as regulation of gene transcription, DNA repair and DNA replication (Lemos and Thakker, 2008). Insight into menin function was obtained by the identification of interacting proteins, which indicate a role in histone modification.

In each cell nucleus, roughly two metres of DNA are packaged into small units called nucleosomes. These nucleosomes consist of the core histone proteins (H2A, H2B, H3 and H4). Menin can influence gene transcription by regulating post-translational modification of the tails of these histone proteins. Menin has been shown to repress gene transcription by attracting histone deacetylase activity (Kim et al., 2003). More recently, menin was found to be an integral component of complexes that contain members of the mixed-lineage leukaemia (MLL) family (Hughes et al., 2004; Yokoyama et al., 2004). MLL proteins possess methyltransferase activity specifically directed at lysine 4 of histone H3 (H3K4), and especially trimethylation of this residue (H3K4me3) (Ruthenburg et al., 2007). Menin is part of the MLL1 and MLL2, but not of the MLL3 or MLL4 complexes (Lee et al., 2006). The menin–MLL1 histone methyltransferase (HMT) complex was found to be important for β-catenin regulated transcription of the *c-Myc* gene and is stabilised by the chromatin-associated protein lens epithelium-derived growth factor (LEDGF) (Sierra et al., 2006; Yokoyama and Cleary, 2008). The menin–MLL1 complex can also activate the expression of several other genes involved in cell proliferation such as the *CDKN2C* and *CDKN1B* cyclin-dependent kinase inhibitor genes and genes involved in cell differentiation such as several homeoboxdomain (Hox) genes (Hughes et al., 2004; Yokoyama et

* Corresponding author at: University Medical Center Utrecht, Department of Physiological Chemistry, STR 3.223, P.O. Box 85060, 3508 AB Utrecht, The Netherlands. Tel.: +31 88 75 68981; fax: +31 88 75 68101.
E-mail address: H.T.M.Timmers@umcutrecht.nl (H.Th.M. Timmers).
[1] Current address: Antonius Ziekenhuis, Nieuwegein, The Netherlands.

0303-7207/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2009.08.020

al., 2004; Milne et al., 2005). It has been shown that loss of menin can lead to reduced H3K4me3 at these specific target genes (Karnik et al., 2005). To date, no study has addressed global H3K4me3 levels in MEN1-related tumours.

The receptor for activated vitamin D ($1,25(OH)_2D_3$; calcitriol) is a member of the nuclear hormone receptor family. In the presence of ligand, the vitamin D receptor (VDR) can bind to vitamin D-responsive DNA elements and regulate transcription of target genes. In parathyroid cells, $1,25(OH)_2D_3$ inhibits parathyroid hormone gene transcription and parathyroid hormone secretion (Demay et al., 1992). Furthermore, $1,25(OH)_2D_3$ negatively regulates parathyroid cell proliferation (Bikle, 2009). Low $1,25(OH)_2D_3$ levels are associated with secondary hyperparathyroidism in patients with renal failure. Several reports suggest that aberrant VDR function can contribute to parathyroid adenoma formation. VDR null or 1α-hydroxylase null (unable to synthesize $1,25(OH)_2D_3$) mice develop parathyroid hyperplasia (Bouillon et al., 2008). VDR gene polymorphisms are associated with primary hyperparathyroidism and alterations of VDR mRNA and protein levels have been reported to occur in parathyroid adenomas (Carling et al., 1995, 2000; Sudhaker Rao et al., 2000). Parathyroid adenomas are the most common manifestation of MEN1, with a penetrance of almost 100%. Moreover, germ line mutations of the *MEN1* gene have been found in patients with familial isolated hyperparathyroidism (FIHP) (Miedlich et al., 2001).

We have previously reported that menin can co-activate nuclear receptor mediated gene transcription (Dreijerink et al., 2006). Reduction of menin levels led to decreased expression of estrogen receptor alpha (ERα) and peroxisome proliferator-activated receptor gamma (PPARγ) target genes (Dreijerink et al., 2006, 2009). To investigate how loss of menin function can lead to aberrant gene expression in MEN1 tumours, we first determined global H3K4me3 staining in parathyroid tumours. To investigate whether expression of specific menin–HMT target genes is affected in MEN1 tumours, we analysed the interaction between menin and VDR and the expression of VDR target genes in MEN1 parathyroid adenomas.

2. Materials and methods

2.1. Patient material

Normal parathyroid tissue and parathyroid adenoma samples were obtained from the Department of Pathology/UMCU biobank and from the Department of Pathology/UMCG and used in accordance with the hospital scientific committee regulations, the Declaration of Helsinki, and the code "Proper Secondary Use of Human Tissue" as installed by the Federation of Biomedical Scientific Societies (http://www.federa.org/?s=1&m=78&p=&v=4).

2.2. Plasmids and mutagenesis

Construction of vectors pXJ440hVDR, pXJ440hVDR(DE), Gal4-RXRα, Gal4-DBD, pEG202NLS-menin and pBabeHygroMenin has been published (Lavigne et al., 1999; Pijnappel et al., 1993; Zwartjes et al., 2004; Hughes et al., 2004; Dreijerink et al., 2006). The menin expression vector pCDNA3.1M+ was a kind gift from G. Weber. The LexA–menin L264P and L267P mutations were introduced by site-directed mutagenesis using the Quikchange procedure. All constructs were verified by DNA sequence analysis of the complete menin cDNA sequence. The B42-hVDR expression plasmid was constructed in the pJG4-5 vector by PCR amplification of the VDR cDNA, from vector pXJ440hVDR using oligos hVDR-EcoR1-F: GATCATCCGAATTCATGGAGGCAATGGCGGCCA and hVDR-Xho1-R: GATCATCCCTCGAGTCAGGAGATCTCATTGCCAAA, and inserted using EcoRI and XhoI.

2.3. Antibodies and immunoblotting

Antibodies used were: anti-menin (Bethyl, A300-105A), anti-H3K4me3 (Abcam, ab-8580), anti-histone H3 (Abcam, ab-1791), anti-Gal4 (Santa Cruz, SC-510) and against HA (Roche, 3F10). Immunoblotting was carried out as described before (Dreijerink et al., 2006).

2.4. Cell lines, stable and transient protein expression

Cos7 (African green monkey kidney) cells and 293T (human embryonic kidney) cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Generation of *MEN1*−/− (MEN1T/T) mouse embryonic fibroblasts (MEFs) has been described (Bertolino et al., 2003). MEFs were maintained in DMEM, containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and 100 μM β-mercaptoethanol. For menin re-expression studies, *MEN1*−/− MEFs were infected with viruses obtained from Phoenix cells transiently transfected with pBabeHygro or pBabeHygroMenin. Cells were grown in medium containing 500 μg/ml hygromycin and monoclonal cell lines were isolated.

Luciferase reporter experiments in Cos7 cells were carried out as described (Dreijerink et al., 2006). In summary, DNA (750 ng per well, in a 12-well format) was transfected using FuGene 6 reagent (Roche). Transfection mixtures consisted of 200 ng luciferase reporter, 25 ng pCMV-*Renilla*, 5 ng pXJ440hVDR(DE), 25 ng Gal4-RXRα, and supplemented with a maximum of 520 ng pCDNA3.1M+ and/or empty pCDNA3 plasmid. 24 h after transfection, the medium was changed to medium containing 100 nM $1,25(OH)_2D_3$ (in ethanol), 1 μM 9-cis-retinoic acid (in ethanol) or vehicle. Luciferase and *Renilla* activities were measured after 24 h. Luciferase assays in MEFs were carried out using Effectine transfection reagent (Qiagen). Transfection mixtures (325 ng per well, in a 12-well format) consisted of 200 ng luciferase reporter, 25 ng pCMV-Renilla, Gal4-DBD, or increasing amounts of pXJ440hVDR(DE), or 50 ng Gal4-p53, supplemented with pCDNA3 plasmid. 24 h after transfection, the medium was changed to medium containing vehicle or 100 nM $1,25(OH)_2D_3$. 24 h later, cells were lysed and luciferase and *Renilla* activities were measured. To be able to compare the results from the two cell lines, results are shown as relative luciferase activities corrected for the ligand-independent signal at the appropriate concentration of Gal4-VDR, to show the fold induction. The Gal4-p53 result was corrected for the Gal4-DBD control experiment.

2.5. Immunohistochemistry

Formalin-fixed (10% (v/v) formalin for 18 h), paraffin-embedded tissues from 2 normal parathyroid glands, 7 sporadic (non-familial) and 4 parathyroid adenomas from MEN1 patients were used. All tissues were sampled from surgical specimens within 2 h after resection. Immunohistochemical staining was essentially performed as previously described using rabbit polyclonal antibodies against menin (dilution 1:100), against H3K4me3 (1:7500) and against histone H3 (1:15,000) (Strik et al., 2002). The optimal dilution for each antibody was determined by end-point titrations on tissue slides of human tonsil. To ensure specific staining the highest dilution was chosen for each antibody, which still yielded a clear nuclear staining. The tissue sections were subjected to antigen retrieval by boiling in 10 mM sodium citrate buffer pH 6 for 15–20 min in a microwave oven. Bound antibodies were visualized with 3,3′-diaminobenzidine (0.1 mg/ml in 0.02% H_2O_2) as the chromogen. Negative control slides were stained with non-immune rabbit IgGs.

Normal, diminished or loss of menin, H3K4me3 and histone H3 staining was determined independently by two experienced pathologists comparing staining intensity of neoplastic cells within the adenoma to the non-neoplastic neuroendocrine cells present in the same slide (often at the rim of the lesion) and to other non-neoplastic cells within the lesion (endothelial and inflammatory cells). The intensity of the staining was scored as: similar (+), weaker (+/−), or negative (−) when the majority of cells (>75%) showed such staining intensity.

2.6. Co-immunoprecipitations

293T cells in 60 mm dishes were transiently transfected with 1 μg of pCDNA3.1M+ or pCDNA3 and 2 μg of pXJ440hVDR or 6 μg of Gal4-DBD. After 24 h the medium was changed to medium containing vehicle or 100 nM $1,25(OH)_2D_3$. After another 24 h cells were lysed in buffer A (50 mM Tris–HCl pH 8, 10% glycerol, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM PMSF, 1% protease inhibitor cocktail (Sigma), 1 mM NaF, 1 mM Na_3VO_4, and 0.5 mM DTT). Dynabeads Protein A was incubated with 2 μg of anti-Gal4 antibody in buffer A for 1 h at 4 °C and then washed 3 times in buffer A before incubating with lysate for 3 h at 4 °C. Subsequently the beads were washed 3 times in buffer A and eluted in sample buffer and proteins retained by the anti-Gal4 antibody were visualized by immunoblot analysis.

2.7. Yeast two-hybrid analysis

EGY48 cells were transformed with the B42-VDR construct and the indicated LexA–menin constructs. Cells were grown overnight at 30 °C in 2% galactose–1% sucrose containing SC medium lacking the appropriate amino acids and in the presence of vehicle or 100 nM $1,25(OH)_2D_3$. Lysates were prepared and the LacZ activity was determined by a liquid β-galactosidase assay as described previously (Albert et al., 2002).

2.8. Analysis of VDR target gene expression

Frozen sections from 6 normal parathyroid tissue samples, 12 sporadic parathyroid adenomas and 7 parathyroid adenomas from MEN1 patients were used for