Menin Induces Apoptosis in Murine Embryonic Fibroblasts*

Received for publication, July 24, 2003, and in revised form, December 18, 2003

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Multiple endocrine neoplasia type I (MEN1) is a hereditary tumor syndrome characterized by multiple endocrine and occasionally non-endocrine tumors. The tumor suppressor gene Men1, which is frequently mutated in MEN1 patients, encodes the nuclear protein menin. Although many tumor suppressor genes are involved in the regulation of apoptosis, it is unclear whether menin facilitates apoptosis. Here we show that ectopic overexpression of menin via adenoviruses induces apoptosis in murine embryonic fibroblasts. The induction of apoptosis depends on Bax and Bak, two proapoptotic proteins. Moreover, loss of menin expression compromises apoptosis induced by UV irradiation and tumor necrosis factor-α (TNF-α), whereas complementation of menin-null cells with menin restores sensitivity to UV- and TNF-α-induced apoptosis. Interestingly, loss of menin reduces the expression of procaspase 8, a critical protease that is essential for apoptosis induced by death-receptor pathways, whereas complementation of the menin-null cells up-regulates the expression of procaspase 8. Furthermore, complementation of menin-null cells with menin increases the activation of caspase 8 in response to TNF-α treatment. These results suggest a pro-apoptotic function for menin that may be important in suppressing the development of MEN1.

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EXPERIMENTAL PROCEDURES

Plasmid Construction—PCR-amplified menin was inserted into the BglII and NotI site of the adenoviral shuttle vector (pAdTrack-CMV) (27) to generate pAdTrack-menin. Plasmids for generating recombinant retroviruses were made by inserting PCR-amplified human menin cDNA into the BamHI/NotI site of the retroviral vector pMX-puro. The constructs were sequenced to verify the fidelity of the sequence.

Production of Recombinant Adenoviruses and Retroviruses—Production of menin-expressing recombinant adenovirus is based on the pAdEasy system, a simplified method for generating recombinant adenovirus (27). Adenoviral titers were determined by plaque assay as well as fluorescence-activated cell scanning (FACS) analysis for percentage of green fluorescent protein (GFP)-positive cells. Retroviruses were
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Preparation of Immortalized Murine Embryonic Fibroblasts (MEFs)—Men1+/− mice (Men1−/−) heterozygous for the Men1 locus were maintained on a 129sv/ESVTac background (Taconic, Germantown, NY) (15). Men1+/− male and female mice were mated, and 9.5 days after plugging the females were euthanized. The embryos were placed in gelatin-coated 12-well plates and dissociated into single cells by repeated pipetting in trypsin buffer. LXS116E6E7 retroviruses that express the human papillomavirus E6 and E7 open reading frame (29) were used to infect the primary MEFs to immortalize the cells. The infected MEFs were subjected to selection with 50 μg/ml G418. Two pairs of menin-null and menin-expressing MEF cell lines (heterozygous and Ad-menin type), each pair derived from littermates, were used. Establishement of Menin-complemented MEF Cell Lines—Immortalized Men1−/− cells (< pass age 12 after isolation from embryos) were seeded in 6-well plates on day 0, infected with vector or menin-expressing retroviruses on day 1, and switched to fresh medium on day 2. Cells were subjected to selection with 2 μg/ml puromycin on day 5 (72 h after switching to fresh medium).

Annexin V and Trypan Blue Staining—For annexin V staining of adenovirus-infected cells, 5 × 10^5 cells were seeded in a 6-well dish and allowed to attach for 5 h. After attachment, cells were infected with adenoviruses (day 0). On the next day (day 1) the medium with adenoviruses was aspirated, and fresh medium was added. On day 2 (24 h after adenovirus infection, fresh medium) cells were harvested. Briefly, cells were collected, stained with annexin V-Cy5 as instructed by the manufacturer (MBL International Inc., Watertown, MA), and analyzed on a BD Biosciences LSR cytometer. The percentage of annexin V-positive cells was determined from gated GFP-positive cell populations.

To examine apoptosis of the menin-null MEFs infected with either vector retroviruses or retroviruses expressing menin, the cells were passaged for ~2 weeks after initial retroviral infection to obtain sufficient numbers of cells for experiments. For trypan blue staining to detect loss of membrane integrity, 8 × 10^5 cells were seeded in a 60-mm dish on day 0. On day 1, cells were treated with or without UV irradiation (100 mJ/m2) by a Spectrolinker XL-1000 (Spectronics Corporation, Westbury, NY) or with or without TNF-α (10 ng/ml, R&D Systems, Minneapolis, MN)/cycloheximide (5.0 μg/ml, Sigma). On day 2 (24 h after the treatment), the cells were collected, stained with trypan blue (0.2%), and counted. Duplicate samples were examined for each data point.

To provide an independent assessment of apoptosis in response to UV or TNF-α treatment, annexin V staining was measured at 16 h after respective treatments. Briefly, 3 × 10^5 cells were seeded/100-mm dish on day 0, treated or not treated on day 1, and harvested 16 h after the treatment on day 2. Cells were collected and stained with annexin V-fluorescein isothiocyante from the MEBCTOB® apoptosis kit as instructed by the manufacturer (MBL International Inc.).

Detection of Release of Free Nucleosomal DNA—Cells were harvested 24–30 h after infection with adenoviruses. The cells were pelleted by centrifugation, and their densities were adjusted to 10^6/ml using the cell lysis buffer from the cell death detection kit (Roche Applied Science). The cell lysate was diluted 10-fold, and 100 μl was used to detect the release of free nucleosomal DNA by enzyme-linked immunosorbent assay based on instructions from the manufacturer.

Western Blotting Analysis—To detect menin expression, whole cell lysates were prepared with whole cell lysis buffer (50 mM Hepes, pH 7.5, 0.4% Triton X-100, 0.1% Nonidet P-40, 150 mM NaCl, 10 mM MgCl2, 0.5 mM EDTA, 2.5 mM EGTA, 0.2 mM Na3VO4, 1 mM NaF, 10 mM β-glycerophosphate, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 4 μg/ml leupeptin, aprotinin, and pepstatin A). Nuclear extracts were prepared as described previously (28). The primary antibody against a human menin peptide (Ser595–Leu610) (30) was raised in rabbits and was affinity-purified with the corresponding peptide-conjugated agarose beads. The bound primary antibodies were detected as described previously (31). To detect procaspase-8 expression, whole cell lysates were prepared from freshly isolated cells using CHAPS lysis buffer (10 mM Tris, pH 7.5, 0.5% CHAPS, 1 mM MgCl2, 1 mM EGTA, 10% glycerol, 1 mM sodium o-vanadate, and 0.1 mg/ml Pefabloc) supplemented with a protease inhibitor mixture (Calbiochem) as described previously (32). Western blotting was performed using a monoclonal antibody from Alexis (ALX-804-448; San Diego, CA).

DNA Microarray and Northern Blotting Analysis—For the microarray, the vector or menin-complemented MEFs were seeded at a density of 2.5 × 10^5 cells/100-mm dish on day 0. The cells were harvested on day 2 for isolation of RNA using the cesium chloride centrifugation method (33). To increase the reproducibility of gene expression profiles, two independent preparations of RNA from each of the cell lines were analyzed. The RNA was processed to generate biotin-labeled RNA probes (34). The probes were hybridized to the Affymetrix murine GeneChip U74A array at the Penn Microarray Core Facility (Philadelphia, PA). Genes whose levels of expression varied 2-fold or more from the control were further analyzed by Northern blotting analysis, which was carried out as described previously (31).

Caspase 8 Enzyme Activity Assay—To compare caspase 8 enzyme activities in vector-complemented and menin-complemented MEFs treated with TNF-α, 3 × 10^5 cells were seeded in 100-mm dishes on day 0. Starting on day 1, TNF-α (10 ng/ml)/cycloheximide (5 μg/ml) was added 0, 3, 6, 12, and 24 h prior to harvesting cells for analysis. Cells were harvested using CHAPS lysis buffer, and protein concentration was determined using BCA reagents (Pierce). The reaction mixtures consisted of the following components: 80 μl of caspase 8 assay buffer (0.1 μM Tris-Cl, pH 8.0, 1 mM EDTA, 1 mM sucrose, 10% glycerol, supplemented with 10 μM dithiothreitol), 100 μg of whole cell lysates, 0.5 μl of Ac-IETD-FAM (Pharmingen), a highly specific caspase 8 substrate, in a final volume of 160 μl. The mixture was incubated for 2 h at 37 °C and measured on a fluorometer with an excitation wavelength of 400 nm and an emission wavelength of 480–520 nm (Bio-Rad). The result from 0 h of TNF-α treatment was set to zero and used to normalize the other measurements. All time points were performed in triplicate.

Statistical Analysis and Quantitation—Standard error bars are noted for appropriate experiments, using Microsoft Excel. Quantitation was performed using either NIH Image 1.63 software (National Institutes of Health, Bethesda, MD) or Imagequant software (Amersham Biosciences).

RESULTS

Adenovirus-mediated Expression of Menin in MEFs Results in Apoptosis—To examine the biological effects of overexpression of menin, we generated menin-expressing adenoviruses (Ad-menin) and control adenoviruses (Ad-GFP). Ad-menin and Ad-GFP, each of which expresses GFP, were used to infect immortalized murine embryonic fibroblasts. Fig. 1A shows that Ad-menin, but not Ad-GFP, causes the majority of infected cells to shrink and detach from the bottom of the plate, characteristic of apoptotic cells. The infection efficiency of Ad-menin and Ad-GFP was similar (Fig. 1A, bottom panels). These results suggest that overexpression of menin results in death of the MEFs.

To examine whether Ad-menin induces apoptosis, the cells were infected with increasing multiplicities of infection (MOIs, from 0 to 20) of either Ad-menin or control Ad-GFP. The infected cells were stained with fluorescent-labeled annexin V, which specifically binds to phosphatidylserine residues that flip from the inner leaflet to the outer leaflet of the plasma membrane during apoptosis (35). The annexin V-stained cells were detected by FACS. Fig. 1B shows that infection of the cells with Ad-menin increases the percentage of the annexin V-stained cells from 7 to 58% (with MOIs from 0 to 20). In contrast, infection of the cells with Ad-GFP only slightly increases this percentage (from 10 to 18%). These results suggest that ectopic expression of menin results in apoptosis in a dose-dependent manner.

Analysis of another marker of apoptosis, release of free nucleosomal DNA (36), confirms the above finding (Fig. 2A). The cells were infected with two independent clones of Ad-menin or Ad-GFP before analyzing the release of free nucleosomal DNA. Fig. 2A shows that Ad-GFP results in only a low level of release of free nucleosomal DNA (0.2–0.3 units), but Ad-menin leads to a marked enhancement of the release of free nucleosomal DNA (0.7–0.9 units). The expression of menin from Ad-menin in the cells was confirmed by immunoblotting using an antimenin antibody (Fig. 2B). Collectively, these results indicate that overexpression of menin, but not GFP, leads to apoptosis in the infected cells. This is consistent with a recent report showing an increased number of annexin V-stained cells in an insulinoma cell line stably overexpressing menin (23).
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Ad-Menin Induces Apoptosis through a Bax/Bak-dependent Apoptotic Pathway—To identify the apoptotic pathway that menin activates and to rule out that overexpression of menin simply results in a nonspecific toxicity, we examined whether ablation of Bax and Bak, two proapoptotic proteins essential for multiple apoptotic pathways (37), blocks Ad-menin-mediated apoptosis. Immortalized wild-type and Bax<sup>−/−</sup>/Bak<sup>−/−</sup> double knock-out (DKO) MEFs (24) were infected with increasing MOIs of either control Ad-GFP-12 or Ad-menin-22 as indicated. The MOIs used for these cells were higher because they are more refractory to infection. The infected cells were harvested, stained with annexin V-Cy5, and analyzed by FACS as described in Fig. 1B. The results are presented as the average of three independent experiments, with S.D. noted. B, the wild-type or DKO cells were infected at an MOI of 200 and processed to detect release of free nucleosomal DNA. The assay was performed in duplicate, with S.D. presented. This is one of two independent experiments. C, the level of menin expression in the infected (MOI of 200) wild-type or DKO cells. Cell lysates (55 μg/lane) from Ad-menin- or Ad-GFP-infected cells were immunoblotted with an antimenin antibody.

Ad-Menin Induces Apoptosis through a Bax/Bak-dependent Apoptotic Pathway—To identify the apoptotic pathway that menin activates and to rule out that overexpression of menin simply results in a nonspecific toxicity, we examined whether ablation of Bax and Bak, two proapoptotic proteins essential for multiple apoptotic pathways (37), blocks Ad-menin-mediated apoptosis. Immortalized wild-type and Bax<sup>−/−</sup>/Bak<sup>−/−</sup> double knock-out (DKO) MEFs (24) were infected with increasing MOIs of Ad-menin or Ad-GFP. In contrast to the shrinkage and detachment we observed in normal cells (Fig. 1A), we saw no such changes in the DKO MEFs (data not shown). To confirm the lack of apoptosis in the DKO cells, the infected cells were stained with annexin V. Analysis by flow cytometry shows that the background annexin V staining accounts for 2–4% of the
control wild-type and DKO cells (Fig. 3A). Infection of the wild-type cells with increasing MOIs of Ad-menin markedly increases the percentage of apoptotic cells (54%), whereas Ad-GFP only slightly increases this percentage (14%, Fig. 3A). In contrast, even at the highest MOI, Ad-menin and Ad-GFP cause similar amounts of apoptosis in DKO cells (13%, Fig. 3A).

In agreement with the above results, infection of wild-type cells with increasing MOIs of Ad-GFP only slightly increases the release of free nucleosomal DNA, from 0.3 to 0.5 units (Fig. 3B, columns 1–4). In contrast, Ad-menin increases the release of free nucleosomal DNA in the wild-type cells from 0.3 to 1.1 units (Fig. 3B, columns 1 and 5–7), indicating a role for menin in inducing apoptosis. Infection of the DKO cells with Ad-GFP does not increase the release of free nucleosomal DNA (Fig. 3B, columns 8–11). In contrast to the wild-type cells, DKO cells infected with Ad-menin do not show cleavage of nucleosomal DNA (Fig. 3B, columns 8 and 12–14), although both the wild-type and DKO cells express a similar level of menin after infection (Fig. 3C). Collectively, these results indicate that menin-mediated apoptosis is dependent on Bax and Bak (25) and that the Ad-menin-induced cell death is not a nonspecific cytotoxicity induced by overexpression of menin.

Complementation of Menin-null MEFs with Menin Sensitizes the Cells to UV- and TNF-α-induced Apoptosis—Next, we determined whether loss of menin expression, which is similar to what occurs in tumors, affects cellular response to apoptosis.

We introduced menin into the immortalized menin-null MEFs by retroviral infection, generating stable cell lines. Fig. 4A shows that menin is expressed in the menin-complemented cells but not in vector-complemented cells. The cells were then treated with UV irradiation, a well-known apoptotic signal, and stained with either trypan blue to detect disruption of cell membrane integrity or annexin V to detect phosphatidylserine translocation. Fig. 4B shows that only 11% of the menin-null cells die after UV treatment, whereas 31% of the menin-complemented cells undergo apoptosis under the same conditions. Consistent with this, only 11% of the vector-complemented cells are annexin V-positive after UV irradiation, whereas 26% of the UV-treated menin-complemented cells are annexin V-positive (Fig. 4C). These results suggest that introduction of menin back into the menin-null cells renders the cells more sensitive to UV-induced apoptosis.

To test whether menin might also be critical for maximal response to other apoptotic signals, we examined whether loss of menin affects the cellular response to TNF-α, which induces apoptosis by binding to death receptors (38). Vector-complemented cells and menin-complemented cells were treated with TNF-α and stained with either trypan blue or annexin V. Fig. 4D shows that only 17% of the vector-complemented cells die after TNF-α treatment, compared with 49% of the menin-complemented cells. Staining for annexin V further confirms these
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Menin Induces the Expression of Procaspase 8 mRNA—As menin is primarily a nuclear protein and is implicated in control of transcription (39, 40), we performed DNA microarray analysis on vector-complemented cells and menin-complemented cells to determine whether any menin-regulated genes are involved in apoptosis. Procaspase 8, a critical protease that is activated by various death receptors that bind TNF-α or Fas ligand (26, 38), was identified as a gene that is up-regulated by menin. Procaspase 8 is essential for apoptosis induced by these death receptors. Upon binding of apoptotic ligands such as TNF-α, the activated death receptors recruit adaptor proteins such as Fas-associated death domain, which in turn binds procaspase 8, leading to generation of active caspase 8 by proteolytic cleavage (26, 38). In addition, several members of the homeobox gene family as well as the insulin growth factor-binding protein family were also found to be differentially regulated by menin in the DNA microarray analysis. However, it is not yet clear whether these members are involved in menin-related apoptosis.

Given the clear role of procaspase 8 in apoptosis, we chose to focus our studies on its regulation by menin. Two pairs of immortalized menin-null cell lines and menin-expressing cell lines, each from a distinct group of littersmates, were established from 9.5-day embryos (Fig. 5A). Expression of menin from these cells was detected as expected, based on genotyping (data not shown) and Western blotting analysis (Fig. 5A). Expression of procaspase 8 mRNA is 2.9-fold higher in menin-expressing cells than in menin-null cells from the first group of littersmates, whereas expression of procaspase 8 is 1.6-fold higher in menin-expressing cells in the second group of littersmates (Fig. 5B, top panel).

To further confirm that menin is responsible for up-regulation of procaspase 8, one of the menin-null cell lines was infected with control retroviruses or viruses expressing menin, and the expression of transduced menin in the infected cells was confirmed by Western blotting analysis (Fig. 6A). Fig. 6B indicates that complementation of the menin-null cells with menin enhances the level of the procaspase 8 mRNA 3.0-fold. Thus, these results indicate that targeted deletion of menin results in decreased levels of the procaspase 8 mRNA (Fig. 5B), whereas restoration of menin increases the levels of the procaspase 8 mRNA (Fig. 6B).
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Menin Induces the Expression of Procaspase 8 Protein as well as Caspase 8 Enzymatic Activity in TNF-α-treated Cells—To examine whether menin-mediated up-regulation of the procaspase 8 mRNA leads to increased expression of procaspase 8, three independent pairs of control cell lines and menin-complemented cell lines were generated. The levels of the procaspase 8 protein from the three pairs were detected by Western blotting analysis. Fig. 7A indicates that complementation with menin enhances expression of the procaspase 8 protein in all three pairs of cell lines. The enhanced levels range from 2.1- to 2.5-fold. In addition, enhanced levels of procaspase 8 may lead to increased caspase 8 activity in response to TNF-α. As shown in Fig. 7B, caspase 8 activity indeed significantly increases in a time-dependent manner in menin-complemented cells in response to the treatment with TNF-α (p < 0.004 24 h after treatment with TNF-α), whereas its activity only slightly increases in the control vector-infected cells. Collectively, these results suggest that menin enhances apoptosis at least in part by up-regulation of procaspase 8 protein and, accordingly, by the caspase 8 activity after activation of the death receptors.

DISCUSSION

Menin, a potent tumor suppressor that suppresses the development of MEN1, interacts with various transcription factors and inhibits the proliferation of oncocytic Ras-transformed cells (20, 39). Although the role of menin in the regulation of cell proliferation has been studied, its role in apoptosis has remained unexplored. The current studies suggest a potentially critical role for menin in promoting apoptosis. Transient overexpression of menin in MEFs induces apoptosis that is dependent on the presence of Bax and Bak, two crucial proapoptotic proteins (Figs. 1–3). Complementation of menin-null MEFs with menin, but not vector, renders the cells more sensitive to both UV- and TNF-α-induced apoptosis (Fig. 4). These findings indicate that extremely high levels of menin induced by recombinant adenoviruses (10× higher than that mediated by retroviral infection; data not shown) can directly cause apoptosis. In contrast, the more physiologically relevant levels of menin induced by retroviruses modulate apoptosis in the presence of apoptotic signals, namely UV and TNF-α. Perhaps transient, extremely high levels of menin resulting from adenovirus infection trigger downstream apoptotic pathways even in the absence of apoptotic stimuli, whereas physiologically relevant levels of menin potentiate apoptosis induced by various apoptotic signals. Similarly, adenovirus-mediated expression of the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome ten) directly results in increased apoptosis in certain cancer cells, whereas endogenous levels of PTEN do not trigger apoptosis (42, 43).

In addition to promoting increased resistance to apoptotic stimuli, loss of the Men1 gene leads to decreased expression of procaspase 8 mRNA, a critical pro tease in death receptor-mediated apoptosis (Fig. 5). Moreover, complementation of menin-null MEFs with menin leads to increased caspase 8 mRNA and protein expression (Figs. 6 and 7). Complementation with menin also leads to increased caspase 8 activation in cells treated with TNF-α, correlating with increased sensitivity to TNF-α. Given the interaction of menin with other transcription factors including JunD, NF-κB, Smad3, and Pem, menin may directly act as a transcriptional co-regulator to modulate the expression of procaspase 8. Alternatively, it may be involved in modifying the promoter of procaspase 8 or the chromatin structure surrounding the promoter. For instance, the promoter of human procaspase 8 has been shown to be methylated in neuroblastoma cells, leading to its decreased expression (45, 46). It is also possible that menin may affect other proteins to indirectly regulate expression of procaspase 8.

The ability of menin to up-regulate caspase 8 expression and its enzymatic activity could help to explain the proapoptotic role of menin in cells treated with TNF-α. TNF-α triggers apoptosis by binding to the TNF-α receptor, which subsequently recruits adapter proteins that lead to the activation of caspase 8 and the initiation of the apoptotic cascade (38). Menin may therefore potentiate the TNF-α-mediated apoptotic signal by increasing the levels of procaspase 8 and its activity. However, it is likely that the modulation of caspase 8 is not the only way in which menin promotes apoptosis. Menin may also modulate the expression of other proapoptotic or antiapoptotic genes to regulate apoptosis. Nevertheless, in the DNA microarray analysis, no other genes related to the caspase 8 pathway were differentially regulated by menin.

In conclusion, these studies indicate a role for menin in promoting apoptosis and the expression of procaspase 8. They suggest apoptosis as another critical function of menin, in addition to inhibition of cell proliferation (20, 23, 47, 48) and involvement in DNA repair (30, 47, 49–51). Attenuation of normal apoptosis in endocrine cells, in combination with increased proliferation and compromised DNA repair capabilities in menin-null cells, may allow cells to accumulate oncogenic mutations, facilitating the development of tumors. Collectively, the current studies suggest a critical role for menin in apoptosis and shed light on the pathogenesis of MEN1.

Acknowledgments—We thank Dr. Sunit Agarwal at NIDDK, National Institutes of Health for the pCMV-Sport-menin construct, Drs. Judy Crabtree and Francis Collins at NHGRI, National Institutes of Health for Men1+/− mice, Dr. Richard Carroll for LXXN16E67 retroviruses, and Dr. J. Alan Diehl for p19Arf−/− MEFs. We are grateful to Dr. Aimee Edinger for assistance with FACS analysis, Drs. Brian Keith and Celeste Simonds for advice on MEF generation. Drs. Thomas and Ting for Bak−/−/Bak−/− DKO cells, and Drs. Xiaolu Yang, David Chang, Hongtu Liu, and Leonardo Salmena for helpful discussions. We thank Dr. Don Baldwin of the Penn Microarray Facility for technical advice and discussions. We thank Drs. Gary Koretzky, Craig Thompson, Michael Brown, and Joseph Goldstein for critically reading the manuscript. We thank Mark Kessler for help in preparing the manuscript.

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J. Biol. Chem. 2004, 279:10685-10691.
doi: 10.1074/jbc.M308073200 originally published online December 18, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308073200

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