Nuclear factor-κB is involved in the protocadherin-10-mediated pro-apoptotic effect in multiple myeloma

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Abstract. The gene encoding protocadherin-10 (PCDH10), a member of the cadherin superfamily, has been recently identified as a tumor suppressor gene (TSG). PCDH10 plays important roles in the apoptosis of tumor cells in some cancer types. However, the exact role of PCDH10 in multiple myeloma (MM) is largely unknown. Increasing evidence has suggested that the activation of nuclear factor-κB (NF-κB) is crucial for apoptosis in myeloma cells. In this study, we investigated the pro-apoptotic effect of PCDH10 on myeloma cells and whether this effect may involve inhibition of the NF-κB pathway. We report here, for the first time to the best of our knowledge, that PCDH10 markedly induces apoptosis of myeloma cells, accompanied by an increase in activated caspase-3 and poly-ADP-ribose polymerase (PARP) levels, and inhibited expression of anti-apoptotic proteins. We also demonstrate that PCDH10 inhibits the activation of NF-κB, by inhibiting the expression of the inhibitor of nuclear factor-κB (IκB) kinase subunits (IKKs) and the phosphorylation of IκBα. Moreover, the constitutive NF-κB DNA-binding activity and the expression of the NF-κB-regulated proteins cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF) and intercellular adhesion molecule 1 (ICAM-1) were inhibited by PCDH10 in MM cells. These results suggest that PCDH10 induces myeloma cell apoptosis, probably by inhibiting the NF-κB pathway.

Introduction

Multiple myeloma (MM) is a hematological malignancy characterized by neoplastic proliferation of monoclonal plasma cells in the bone marrow (1). MM represents ~13% of hematological malignancies and 2% of all cancer types (2). Despite the use of high-dose chemotherapeutic agents combined with hematopoietic stem cell transplantation, MM remains incurable, due to its unclear pathogenesis, the lack of novel potent therapies and also, increasing chemoresistance (3). One approach to significantly enhance the survival rate of MM patients is the identification of novel therapies that involve targeting of the deregulated signaling pathways that contribute to chemoresistance (4). Inhibition of nuclear factor-κB (NF-κB) signaling may allow to overcome chemoresistance in patients with MM, while inhibition of this transcription factor also remains a compelling approach in the development of therapies to treat the disease (5).

Protocadherin-10 (PCDH10, also known as OL-PCDH or KIAA1400) belongs to the δ subgroup of the protocadherin subfamily; the gene locates on the human chromosome 4q 28.3 (6). Previous studies indicated that PCDH10 may be a tumor suppressor gene (TSG). Its expression was downregulated or suppressed in numerous human cancer types including MM, and gastric and cervical cancer, and the reduced expression of the PCDH10 gene was found to be due to methylation of its promoter (7-17). Therefore, restoring the expression of PCDH10 inhibited cell growth, migration, invasion and colony formation of tumor cells (15), but there have been few reports investigating the exact underlying mechanisms in tumor cells. Our previous study indicated that PCDH10 is totally silenced due to promoter methylation in the KM3 and RPMI-8226 cell lines, and that ectopic expression of the gene inhibits cell proliferation and angiogenesis (8). However, the effect of PCDH10 on apoptosis of MM cells and the underlying mechanism have not yet been reported. In addition, it has been shown that PCDH10 exerts pro-apoptotic effects in gastric cancer by upregulating pro-apoptotic genes including Fas, caspase-8, Jun and CDKN1A (17). Therefore, we hypothesized that PCDH10 might also induce apoptosis in MM cells.

Since the survival and proliferation of myeloma cells are supported by growth factors that signal through cell surface receptors that activate the NF-κB pathway, the NF-κB

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signaling pathway is crucial in the pathogenesis and treatment of MM (18,19). Numerous studies have provided evidence that NF-κB mediates a number of events in carcinogenesis including deregulation of cell proliferation, tumor invasion, metastasis and angiogenesis (20). It has also been shown that NF-κB is constitutively active in myeloma cells, and that suppression of NF-κB exerts pro-apoptotic effects on myeloma cells (21). In addition, conventional and novel anti-myeloma agents for MM including dexamethasone, thalidomide and bortezomib interfere with the activation of NF-κB (22,23). Based on these studies, NF-κB is an important therapeutic target for MM treatment, and therefore, various agents that block the NF-κB pathway are prospective candidates for treatment of human myeloma.

In the present study, we hypothesized that PCDH10 may exert a pro-apoptotic effect on myeloma cells through downregulation of the NF-κB pathway. To investigate this hypothesis, we examined whether PCDH10 promotes apoptosis and inhibits the NF-κB signaling pathway in MM cells, and evaluated the therapeutic potential of PCDH10 in MM cells in vitro.

Materials and methods

Antibodies. The antibodies targeting NF-κB p65, phosphorylated (p)IκBα, inhibitor of nuclear factor κB (IκB) kinase subunit (IκKα, IκKβ), pIkBα, goat anti-mouse horseradish peroxidase (HRP)- and goat anti-rabbit HRP-conjugated antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibodies targeting PCDH10, survivin, induced myeloid leukemia cell differentiation protein (Mcl-1), B-cell lymphoma (Bcl)-2, intercellular adhesion molecule-1 (ICAM-1), cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), β-actin and Cy3-labeled goat anti-rabbit anti-IgG were purchased from Beyotime Institute of Biotechnology (Jiangsu, China) were maintained in Gibco (Life Technologies, Grand Island, NY, USA). The plasmid clone KIAA1400 (a gift from the Kazusa DNA Research Institute, Japan) using the AccuPrime Pfx DNA polymerase (Life Technologies) according to the manufacturer's instructions.

Identification of MM cells stably expressing PCDH10. The cells were transferred to new plates 48 h following transfection, and 400 µg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium; screening of resistant cells was performed 21 days later. Expression of PCDH10 in the resistant cells was confirmed by RT-PCR and western blotting.

Total RNA isolation and semi-quantitative reverse transcription-PCR (RT-PCR). Total RNA was extracted using the TRIzol reagent (Life Technologies). cDNA was then synthesized using the GoTaq polymerase (Promega, Madison, WI, USA) and random hexamer primers. The housekeeping gene encoding β-actin served as the loading control. The primers used for amplification were: PCDH10 forward (F), 5'-ACT GCT ATC AGG TAT GCC TG-3'; and reverse (R), 5'-GTC TGT TGA CTA GAT AGC TG-3'; β-actin F, 5'-CTC CAT CCT GGC CTC GCT GT-3'; and R, 5'-GTC GCT ACC TTC ACC GTT CC-3'. Amplification of PCDH10 was performed for 32 cycles and that of β-actin for 23 cycles.

Protein extraction and western blot analysis. Western blot analysis was performed in PCDH10-transfected cells as previously described (24) in order to detect the protein levels of: IKKα and β and pIkBα in the cytoplasm; pp65 in the nucleus; and procaspase-3, cleaved caspase-3, PARP, Mcl-1, Bcl-2, Bcl-xL, survivin, XIAP, cIAP-1, cIAP-2, ICAM-1, COX-2 and VEGF in whole-cell extracts. The total protein content of the cells was extracted using the M-PER mammalian protein extraction reagent (Pierce, Rockford, IL, USA) supplemented with protease and phosphatase inhibitors, following the manufacturer's instructions. Extraction of cytoplasmic and nuclear proteins was performed using BeyoECL Plus nuclear and cytoplasmic protein extraction kits (Beyotime Institute of Biotechnology, Jiangsu, China). Protein concentrations were measured with the bicinchoninic acid (BCA) method using the BCA protein assay reagent kit (Pierce). The gel-separated proteins (50-80 µg of protein/lane) were then electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). After blocking with 5% non-fat milk for 1 h, membranes were incubated overnight at 4°C with the respective primary antibodies. After washing, membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Blots were then developed with enhanced chemiluminescence (ECL; Beyotime Institute of Biotechnology). Immunoblotting with anti-β-actin confirmed equal protein loading.

Cell viability assay. Stably-transfected clones of RPMI-8226 and KM3 cells expressing PCDH10 were selected as described above. Two clones of each cell line were multiplicated and used for the assay. The clones were seeded into 96-well plates. The colorimetric MTT assay (Sigma-Aldrich) was used to measure cell numbers at the special time points. The experiment was performed three times in 6-well replicates.
Apoptotic assay. The percentage of apoptotic cells was determined using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Bender Medsystems, Burlingame, CA, USA) as previously described (25). PCDH10-transfected MM cells were collected, washed and resuspended in 500 µl of binding buffer containing FITC-conjugated Annexin V and propidium iodide (PI). Following incubation for 30 min at room temperature in the dark, the cells were analyzed immediately by flow cytometry (FACS Vantage SE, Becton, Dickinson, San José, CA, USA).

Morphological assessment of apoptotic cells was performed with transmission electron microscopy (TEM). MM cells were harvested and fixed with 1% osmium tetroxide (Spectrum, Chino, CA, USA) for 1 h. Samples were then dehydrated through incubation in a graded ethanol series followed by a graded-series incubation in 99% propylene oxide (Hengshui Taocheng Chemical Auxiliary Co., Ltd, Hengshui, China). The samples were then infiltrated overnight in a 1:1 mixture of propylene oxide and epoxy resin (Truetime Industrial Corporation, Taiwan, China). The following day, cells were infiltrated for 8 h before embedment in fresh resin. Areas selected for ultramicrotomy were sectioned at 70-90 nm with a diamond knife and placed on 300-mesh copper grids. Sections were stained with uranyl acetate (Truetime Industrial Corporation) for 1 h and lead citrate for 2-3 min. All sections were examined by TEM using the TM8-H-7500 microscope (Hitachi, Tokyo, Japan).

Detection of NF-κB p65 by immunofluorescence. The localization of NF-κB was examined in MM cell lines by immunofluorescence as previously described (26). PCDH10-transfected cells were applied onto ice-cold microscope slides, air dried for 12 h at room temperature and fixed with cold acetone. Following a brief washing in phosphate-buffered saline, slides were blocked with 5% normal goat serum for 1 h and then incubated with anti-NF-κB p65 (dilution, 1:100) overnight at 4°C. The slides were washed, incubated with Cy3-labeled goat anti-rabbit anti-IgG (dilution, 1:200) for 1 h, and the nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich) for 5 min. Stained slides were observed under a fluorescent microscope Olympus 1X71 fluorescence microscope (Olympus, Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA). The DNA-binding activity of NF-κB was measured in MM cell lines by immunofluorescence as previously described (27). PCDH10-transfected cells were collected and resuspended in 500 µl of binding buffer containing FITC-conjugated Annexin V and propidium iodide (PI). Following incubation for 30 min at room temperature in the dark, the cells were analyzed immediately by flow cytometry (FACS Vantage SE, Becton, Dickinson, San José, CA, USA).

Results

PCDH10 reduces tumor cell viability. To evaluate the effect of PCDH10 on MM cell growth, the RPMI-8226 and KM3 lines, in which PCDH10 is fully silenced by methylation, were transfected with the expression vector encoding the full-length PCDH10 or with the empty vector. After selection in G418-supplemented medium for 3 weeks, stable expression of PCDH10 was confirmed by RT-PCR and western blotting in the MM lines (Fig. 1A).

The cell viability assay was performed in stably transfected RPMI-8226 and KM3 cells. The cells that were transfected with pcDNA3.1(+)+/PCDH10 grew significantly slower than the empty vector-transfected cells (P<0.05) (Fig. 1B), indicating that PCDH10 reduces tumor cell viability.

PCDH10 induces apoptosis in MM cell lines. In order to investigate whether PCDH10 exerts an apoptotic effect on myeloma cells, we examined cell apoptosis by Annexin V-FITC and PI staining. We found that PCDH10 strongly increased the...
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percentage of apoptotic (Annexin V-positive) MM cells. Following transfection with the PCDH10 gene, the percentage of Annexin V-positive cells was estimated at 18.37% in RPMI-8226 cells and at 11.14% in KM3 cells (Fig. 2B); these percentages were significantly higher than those of the untreated or the vector-transfected group (Fig. 2C).
The induction of apoptosis in MM by PCDH10 was also confirmed by TEM. The morphology of apoptotic MM cell nuclei was characterized by extensive chromatin condensation and membrane blebbing, whereas control cells showed limited or no signs of apoptosis (Fig. 2A).

PCDH10 activates caspase-3 and PARP in MM cells. Caspase-3 is a key effector caspase, involved in the proteolytic cleavage of numerous proteins during apoptosis, such as PARP (27). In this study, we found an increase in the level of cleaved caspase-3 and PARP in MM cells transfected with PCDH10 (Fig. 3A). These results suggested that the pro-apoptotic effect of PCDH10 on MM cells at least partly involves the activation of caspases.

PCDH10 decreases the expression level of anti-apoptotic proteins in MM cells. To elucidate the molecular mechanism of PCDH10-induced cell apoptosis in MM cells, we examined the expression levels of the anti-apoptotic Bcl-2 and IAP family members, which block the cell apoptotic pathway (28,29). Western blot analysis revealed that transfection with PCDH10 decreased the expression of Mcl-1, Bcl-xL, Bcl-2, survivin, XIAP and cIAP-1, but not that of cIAP-2 (Fig. 3B). These results suggested that PCDH10 may induce myeloma cell apoptosis through the downregulation of genes encoding anti-apoptotic factors.

PCDH10 inhibits the constitutively active NF-κB protein in MM cells. NF-κB can regulate the expression of anti-apoptotic proteins such as Bcl-2, Bcl-xL, survivin, XIAP, cIAP-1, -2 and Mcl-1. Since we found that PCDH10 decreases the expression of these proteins, we further investigated the effect of PCDH10 on NF-κB activation. The subcellular localization of NF-κB in MM cells was assessed by western blot analysis. We found that PCDH10 blocked the phosphorylation of NF-κB p65 in the RPMI-8226 and KM3 lines (Fig. 4A). When NF-κB is activated, p65 is translocated from the cytoplasm into the nucleus. To confirm whether PCDH10 inhibits the translocation of p65 in the nucleus, we further examined by immunofluorescence the intracellular distribution of p65 in RPMI-8226 and KM3 cells following transfection with PCDH10. An important decrease in the relative level of p65

Figure 4. Protocadherin-10 (PCDH10) reduces the level of the constitutively activated nuclear factor-κB (NF-κB) protein in multiple myeloma (MM) cell lines. (A) PCDH10 reduces the level of phosphorylated (p)NF-κB p65 in RPMI-8226 and KM3 cells transfected with PCDH10. pNF-κB p65 (Ser536) was detected by western blot in whole-nuclear extracts; β-actin was used as an internal loading control. (B) PCDH10 inhibits the expression of the phosphorylated form of the inhibitor of nuclear factor-kB (IκB)α. Cytoplasmic extracts were prepared to detect the expression of pIκBα (Ser32) by western blotting. (C) PCDH10 blocks the translocation of p65 to nucleus. The intracellular distribution of p65 was examined in PCDH10-expressing MM cells (2x10⁶) by immunofluorescence (magnification, x400) as described in Materials and methods. The nuclei were stained blue with DAPI, the NF-κB p65 protein was stained green, and the superposition of the two stained fields is shown in the Merge panel. (D) PCDH10 reduces the expression of the IkB kinase subunit (IKKα and β). Cytoplasmic extracts were prepared to detect the expression of IKKα and β by western blot. The pictures are representative blots from 3 independent experiments. (E) The constitutive DNA-binding activity of NF-κB is significantly inhibited by PCDH10 in MM cells. The DNA-binding activity of the transcription factor NF-κB was quantified in MM cells by the TransAM® NF-κB p65 enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer’s instructions. Mean values were normalized for cellular protein content. Bars denote SD from 3 experiments. *P<0.05 compared to the Vector group. Mock, untreated cells; Vector, empty vector pcDNA3.1(+).
in the nucleus and a marked increase in the cytoplasm was observed in PCDH10-transfected MM cells (Fig. 4C). As shown in Fig. 4E, constitutive NF-κB DNA-binding activity was significantly inhibited by PCDH10 in MM cells (P<0.05).

**PCDH10 inhibits the expression of IKKs and inhibits phosphorylation of IκBα in MM cells.** To explore whether the inhibition of NF-κB activation by PCDH10 is caused by inhibition of IKKs, we examined the expression of IKKα, IKKβ and the phosphorylation of IκBα in the cytoplasm following transfection with PCDH10. We found that PCDH10 reduces the expression of IKKα and IKKβ in MM cells (Fig. 4D). Furthermore, the level of phosphorylated IκBα was also decreased in PCDH10-transfected MM cells (Fig. 4B).

**PCDH10 reduces the expression level of NF-κB-regulated proteins in MM cells.** Activation of NF-κB is known to induce the expression of ICAM-1, COX-2 and VEGF (20). We therefore investigated the effect of PCDH10 on the expression of these proteins by western blotting. We observed that PCDH10 reduced the expression levels of ICAM-1, COX-2 and VEGF (Fig. 5).

**Discussion**

The present study aimed to investigate the pro-apoptotic effect and the underlying mechanism of action of PCDH10 in MM cells. This is the first study, to the best of our knowledge, that demonstrates that PCDH10, encoded by a novel TSG, strongly induces apoptosis of MM cells, and that this effect associates with activation of caspase-3 and PARP and with inhibition of the expression of anti-apoptotic proteins regulated by NF-κB. These findings suggest that the pro-apoptotic effect of PCDH10 is mediated at least in part by the inhibition of the constitutive activation of NF-κB in MM.

Little is known on the function of PCDH10 in MM, apart from a study by Li et al (8), which showed that PCDH10 represents a TSG by transfecting MM cells with a PCDH10-expression plasmid. G1 cell cycle arrest and suppressed colony formation upon reversal of the epigenetic silencing of PCDH10 were also reported in this study. Thus, we hypothesized that PCDH10 might induce apoptosis in MM cells. In line with this hypothesis, we showed that restoring the expression of PCDH10 exerts a considerable pro-apoptotic effect in both RPMI-8226 and KM3 cell lines. These results are in agreement with previous findings on PCDH10 in gastric cancer cells (17).

It is well established that caspase-3 is a critical effector of apoptosis, since it is either partially or exclusively responsible for the proteolytic cleavage of the nuclear enzyme PARP (27). Since NF-κB acts as an anti-apoptotic factor in MM, by regulating caspase activation through a number of mechanisms, we next examined whether PCDH10 induces apoptosis via caspase activation. As expected, we found that expression of PCDH10 leads to cleavage of procaspase-3 to caspase-3, and caspase-3-regulated cleavage of PARP. Since various pro-apoptotic and anti-apoptotic proteins play critical roles in the regulation of apoptosis (27-29), we further investigated whether the expression of these proteins is altered by PCDH10 using western blot analysis. The results indicated that PCDH10-induced apoptosis of myeloma cells is associated with decreased expression of a number of anti-apoptotic proteins, including Bcl-2-related family members (Mcl-1, Bcl-2 and Bcl-xL) and IAP family members (survivin, XIAP and cIAP-1); this may explain the PCDH10-mediated activation of myeloma cell apoptosis.

It has been reported that the activation of NF-κB contributes to the pathogenesis of MM via egulation of the expression of growth factors, anti-apoptotic genes, and proteins that are involved in angiogenesis. We found that PCDH10 promotes apoptosis of MM cells by reducing the expression level of Bcl-2 and IAP family members that are directly regulated by NF-κB. Based on this finding, we next examined whether PCDH10 induces cell apoptosis by blocking the activation of NF-κB in MM cells. Using RPMI-8226 and KM3 cells that constitutively express active NF-κB, we found that PCDH10 inhibits the phosphorylation of NF-κB and its translocation to the nucleus. The expression of anti-apoptotic proteins (Mcl-1, Bcl-2, Bcl-xL, survivin, XIAP and cIAP-1) has been shown to be regulated by NF-κB (30-32). Considering these studies, our results confirm that PCDH10-induced inhibition of the expression of these proteins may partly depend on the inhibition of the NF-κB activity.

In addition, our study indicated that PCDH10 inhibits NF-κB activation through inhibition of IKKs, which have been shown to phosphorylate NF-κB (33). We also found that reduced expression of IKKs in PCDH10-expressing cells was associated with reduced phosphorylation of IκBα, which is an NF-κB inhibitor. These findings further support that the pro-apoptotic activity of PCDH10 is mediated at least in part by the NF-κB pathway. Future studies are needed to further elucidate the exact molecular interaction between PCDH10 and NF-κB in the context of MM.

The expression of the anti-apoptotic proteins ICAM-1, COX-2 and VEGF, which are regulated by NF-κB (20), was...
found to be inhibited by PCDH10 using western blotting. Downregulation of the expression of these gene products might be the result of inhibition of NF-xB by PCDH10. Furthermore, constitutive NF-xB DNA-binding activity in MM cells was significantly inhibited in PCDH10-expressing cells, which may explain the observed inhibition of NF-xB. Consequently, these results support our hypothesis that PCDH10 can block NF-xB activation.

It is notable that a recent study showed that the binding sites for NF-Y and Sp1/Sp3 are critical for the transcription, and thus the expression and function, of PCDH10; thus, the levels or activities of these two transcription factors may modulate PCDH10 expression (10). Additional work is required to further clarify how PCDH10 functions as a TSG in MM. Whether PCDH10 is dependent on the expression of other proteins is an issue that will be investigated in future studies.

In conclusion, our results show for the first time, to the best of our knowledge, that PCDH10 can induce apoptosis of MM cells. The pro-apoptotic effect of PCDH10 is mediated by activation of caspase-3 and PARP and downregulation of the anti-apoptotic proteins Mcl-1, Bcl-2, Bcl-xL, survivin, XIAP and cIAP-1. The downregulation of these proteins may be due to the inhibition of the NF-xB pathway in vitro. Our study provided a foundation for clinical trials of demethylation drugs for myeloma and a rationale for their use in combination with therapeutic agents, particularly bortezomib. It is however necessary to further clarify the roles of PCDH10 in regulation of apoptosis in MM cells, along with the precise molecular mechanism(s) underlying this effect.

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