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Transglutaminase-2 Is Involved in Expression of Osteoprotegerin in MG-63 Osteosarcoma Cells

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Abstract

Osteoprotegerin (OPG) is a secreted glycoprotein and a member of the tumor necrosis factor receptor superfamily. It usually functions in bone remodeling, by inhibiting osteoclastogenesis through interaction with a receptor activator of the nuclear factor κB (RANKL). Transglutaminases-2 (Tgase-2) is a group of multifunctional enzymes that plays a role in cancer cell metastasis and bone formation. However, relationship between OPG and Tgase-2 is not studied. Therefore, we investigated the involvement of 12-O-Tetradecanoylphorbol 13-acetate in the expression of OPG in MG-63 osteosarcoma cells. Interleukin-1β time-dependently induced OPG and Tgase-2 expression in cell lysates and media of the MG-63 cells by a Western blot. Additional 110 kda band was found in the media of MG-63 cells. 12-O-Tetradecanoylphorbol 13-acetate also induced OPG and Tgase-2 expression. However, an 110 kda band was not found in TPA-treated media of MG-63 cells. Cystamine, a Tgase-2 inhibitor, dose-dependently suppressed the expression of OPG in MG-63 cells. Gene silencing of Tgase-2 also significantly suppressed the expression of OPG in MG-63 cells. Next, we examined whether a band of 110 kda of OPG contains an isopeptide bond, an indication of Tgase-2 action, by monoclonal antibody specific for the isopeptide bond. However, we could not find the isopeptide bond at 110 kda but 77 kda, which is believed to be the band position of Tgase-2. This suggested that 110 kda is not the direct product of Tgase-2's action. All together, OPG and Tgase-2 is induced by IL-1β or TPA in MG-63 cells and Tgase-2 is involved in OPG expression in MG-63 cells.

Key Words: Osteoprotegerin, Transglutaminase-2, MG-63 cell, Cystamine, IL-1β, TPA

INTRODUCTION

Osteoprotegerin (OPG) is a secreted glycoprotein belonging to the tumour necrosis factor receptor (TNFR) superfamily (Simonet *et al.*, 1997; Tsuda *et al.*, 1997). OPG is central in the regulation of bone turnover through the inhibition of osteoclastogenesis (Tsuda *et al.*, 1997). OPG is absent of a transmembrane domain, making this a decoy receptor with the ability to bind a number of different ligands, and is produced as a monomer (55-62 kDa). It is secreted as a disulfide-linked homo dimeric glycoprotein with four or five potential glycosylation sites, generating a mature form (110-120 kDa). OPG functions both in bone remodeling, by inhibiting osteoclastogenesis through its interaction with receptor activator of the nuclear factor κB, and in survival, by acting as a decoy receptor for TNF-related apoptosis-inducing ligand, preventing its interaction with the functional death receptors; thus, allowing cells to escape cell death (Emery *et al.*, 1998; Yasuda *et al.*, 1999). Furthermore, recently, OPG is also reported as a marker for several diseases, including marker of atherosclerosis in diabetic patients (Zauli *et al.*, 2009; Augoulea *et al.*, 2013). Recently, a high level of OPG was an independent risk marker of all-cause mortality in a high-risk population of hemodialysis patients with previously documented cardiovascular disease and OPG is also regarded as one of biochemical markers of vascular calcification (Osorio *et al.*, 2013; Winther *et al.*, 2013).

Transglutaminase-2 (Tgase-2) is a multifunctional protein with both intracellular and extracellular functions (Lee *et al.*, 2012). In addition to catalyzing Ca^{2+}-dependent transamidation reactions, Tgase-2 can bind and hydrolyze GTP/GDP with a similar affinity and catalytic rate to the α subunit of large heterotrimeric G proteins and small Ras-type G proteins (Lorand and Graham, 2003; Mhaouty-Kodja, 2004; Lee *et al.*, 2012). Tgase-2 activates NF-κB via polymerization of I-κB (Lee *et al.*, 2012). Recently, we also showed that Tgase-2 is involved in JNK activation and PP2A downregulation (Park *et al.*, 2011; Park *et al.*, 2012). Transamidation activity of Tgase is in-

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creased in osteoarthritis (OA) joint cartilage (Rosenthal *et al.*, 1997). Tgase-2 is also expressed hypertrophic chondrocytes (Nurminskaya *et al.*, 2003). Tgase-2 is also an essential mediator of Interleukin-1β (IL-1β)-induced calcification, as well as hypertrophic differentiation and calcification in articular chondrocytes *in vivo* and *in vitro* (Johnson and Terkeltaub, 2005). Tgase-2 is described as a biomarker of OA severity in Hartley guinea pig knees (Johnson *et al.*, 2004) Tgase-2 is central to induction of the arterial calcification (Johnson *et al.*, 2008).

Several inflammatory mediators, including IL-1β, TNF-α and TGF-β, induce OPG expression (Brandstrom *et al.*, 1998; Vidal *et al.*, 1998; Thirunavukkarasu *et al.*, 2001). Both p38 and ERK signaling pathways are involved in OPG expression and involvement of NF-κB is still controversial (Kobayashi-Sakamoto *et al.*, 2004; Lambert *et al.*, 2007; McCarthy *et al.*, 2009). Therefore, details of signaling pathways of OPG are still unclear. Moreover, both of Tgase-2 and OPG are involved in calcification, relationship between OPG and Tgase-2 is not studied. Especially involvement of Tgase-2 in OPG expression is not reported.

In this report, we showed that Tgase-2 is induced in IL-1β or TPA-treated MG-63 cells with concomitant induction of OPG, and that Tgase-2 is involved in OPG expression.

MATERIALS AND METHODS

Materials

Recombinant human IL-1β was purchased from the R&D systems, Inc. (St. Louis, MO, USA). A 12-O-Tetradecanoylphorbol 13-acetate (TPA) and cystamine (CTM) were purchased from Sigma Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and DMEM were obtained from WelGENE Inc. (Daegu, South Korea). All other chemicals were of standard analytical grade. Antibody against OPG was obtained from the R&D systems, Inc. (St. Louis, MO, USA), antibody to Tgase-2 was from NeoMarkers (Fremont, CA, USA) and antibody to β-actin was from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

Human osteosarcoma MG-63 cells were purchased from ATCC (Rockville, MD, USA). The cells cultured in DMEM supplemented with 10% (v/v) heat-activated fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 U/ml) at 37°C in a 5% CO_2 atmosphere.

Protein extracts from culture media or cells

Cell culture supernatants were collected and centrifuged at 1,000 rpm, 5 min. The cell free supernatant was incubated with 80% cold acetone at -20°C for 60 min. Subsequently, the samples were centrifuged at 15,000 rpm at 4°C for 30 minutes. The supernatants were carefully aspirated and the pellets were allowed to air dry at 23°C.

For whole-cell lysate, the cells were washed twice with cold PBS. The cells were lysed in a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% triton X-100, 2 mM EDTA, 1% DOC (Deoxycholic acid), 0.1% SDS, 1 mM $NaVO_3$, 10 mM NaF, 1 mM DTT] and centrifuged to yield whole-cell lysates. Protein concentration was measured using the Bradford method.

Western blot analysis

Aliquots of the lysates (20-30 μg of protein) were separated on a 4-12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Invitrogen, Carlsbad, CA, USA) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 10% MeOH (v/v)]. After blocking the nonspecific site with 5% non-fat dry milk, the membrane was then incubated with specific primary antibody in 3% BSA at 4°C overnight. The membrane was further incubated for 60 min with a peroxidase-conjugated secondary antibody (1:2,000, Santa Cruz, CA, USA) at room temperature. Immunoactive proteins were detected using the WEST-ZOL (plus) Western Blot Detection System (iNtRON, Gyeonggi, Korea).

Cell proliferation assay

Cell proliferation was measured using the EZ-Cytox Cell viability assay kit (Daeillab service, Seoul, Korea). Briefly, 100 μl of cell suspension (3,000 cells per well) was added into each well of a 96-well plate. After the required incubation with the stimulants for indicated time, 10 μl of EZ-Cytox solution was added to each well of the plate and incubated at 37°C for 2 h. The absorbance was measured by a spectrophotometer (Multscan, Thermo, USA) at 450 nm. The cell proliferation (%) was calculated using the formula: [As/Ac]×%. As: the absorbance of well containing cell, culture medium, EZ-Cytox solution and stimulants; Ac: the absorbance of well containing cell, culture medium and EZ-Cytox solution.

Tgase 2 gene silencing by small interfering RNA

A small interfering RNA (siRNA) duplex targeting human Tgase 2,5'-AAGAGCGAGAUGAUCUGGAAC-3' (Invitrogen) was introduced into the cells, using Lipofectamine 2,000 (Invitrogen), according to the manufacturer's instruction. Forty-eight hours after transfection, the cells were harvested, and a cytosolic faction was prepared in order to analyze the level of Tgase 2 and OPG by Western blotting. Cells incubated with Lipofectamine 2,000 and Stealth Negative control (Invitrogen) were employed as the negative control.

RESULTS

IL-1β increased Tgase-2 and OPG expression in MG-63 osteosarcoma cells

OPG is induced by several cytokines, such as IL-1β and TNF-α (Vidal *et al.*, 1998; Thirunavukkarasu *et al.*, 2001). We chose IL-1β as an inducer. In order to investigate the expression of OPG in IL-1β-induced osteosarcoma cell line, MG-63 cells were treated with IL-1β for the indicated time. IL-1β time-dependently induced OPG and Tgase-2 expression in cell lysates and media of MG-63 cells as shown by a Western blot analysis (Fig. 1). Additional 110 kda band was found in the media of MG-63 cells (Fig. 1B).

TPA increased Tgase-2 and OPG expression in MG-63 osteosarcoma cells

OPG is reported to be induced by TPA (Kondo *et al.*, 2002). Therefore, we also examined the effect of TPA on the expression of OPG and Tgase-2 in MG-63 cells. TPA also time-dependently induced OPG and Tgase-2 expression (Fig. 2A). In contrast to media of IL-1β treated MG-63 cells, a 110 kda band was not found in TPA-treated media of MG-63

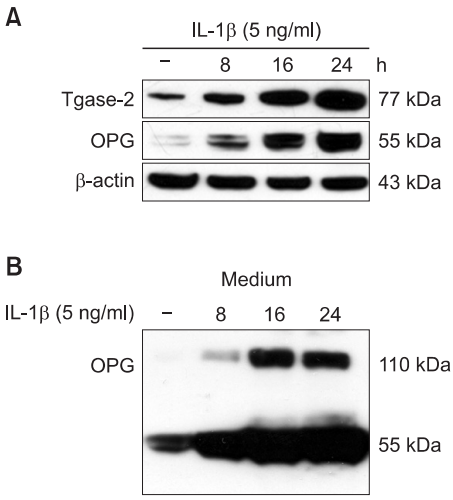

Fig. 1. IL-1β increased Tgase-2 and OPG expression in MG-63 osteosarcoma cells. (A) IL-1β time-dependently induced OPG and Tgase-2 expression in cell lysates. MG-63 cells were treated with control media or IL-1β (5 ng/ml) for indicated time. Whole-cell lysates were subjected to 4-12% SDS-PAGE, and expression of Tgase-2 and OPG were determined by Western blotting. β-actin was used here as an internal control. (B) IL-1β time-dependently induced 110 kDa OPG expressions in the cell media. Culture media were subjected to 4-12% SDS-PAGE, and the expression of OPG was determined by Western blotting.

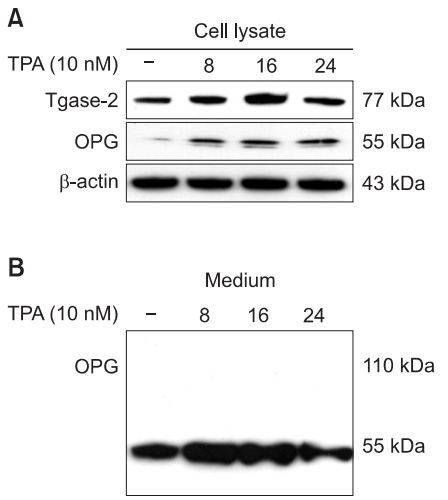

Fig. 2. TPA increased Tgase-2 and OPG expression in MG-63 osteosarcoma cells. (A) TPA induced OPG and Tgase-2 expression in cell lysates. MG-63 cells were treated with control media or TPA (10 nM) for the indicated time. Whole-cell lysates were subjected to 4-12% SDS-PAGE, and expression of Tgase-2 and OPG were determined by Western blotting. β-actin was used here as an internal control. (B) TPA induced OPG expressions in the cell media. Culture mediums were subjected to 4-12% SDS-PAGE, and expression of OPG was determined by Western blotting.

cells (Fig. 2B). The expression was not strong as seen with IL-1β.

CTM and gene silencing of Tgase-2 suppressed IL-1β-induced OPG expression in MG-63 osteosarcoma cells

To investigate the role of Tgase-2 in OPG expression, we examined the effects of CTM, well known inhibitor, on the OPG expression in MG-63 cells. CTM dose-dependently suppressed the IL-1β-induced OPG expression of MG-63 cells (Fig. 3A). CTM also dose-dependently suppressed the TPA-induced OPG expression of MG-63 cells (Fig. 3B). To confirm the involvement of Tgase-2, we also examined the involvement of Tgase-2 in OPG expression by gene silencing of Tgase-2. To be consistent with the effects of CTM, gene silencing of Tgase-2 also significantly suppressed the expression of Tgase-2 in MG-63 cells (Fig. 3C).

110 kDa protein is not detected by isopeptide specific monoclonal antibody

Tgase-2 is known to selectively cross-link osteopontin by isopeptide bonding (Higashikawa *et al.*, 2007). Therefore, we examined whether the IL-1β-induced Tgase-2 crosslink the OPG protein using monoclonal antibody specific for isopeptide bond. However, we could not find the isopeptide bond at 110 kda but detect 77 kda, which is believed to be the band position of Tgase-2 (Fig. 4).

DISCUSSION

OPG is a soluble member of the tumor necrosis factor receptor superfamily, which potently inhibits RANKL-mediated osteoclastogenesis (Zauli *et al.*, 2009). The administration of OPG-Fc counteracted bone loss in several preclinical models of cancers. In addition, several *in vitro* studies reported that full-length OPG also antagonizes the death-inducing ligand TRAIL (Emery *et al.*, 1998; Truneh *et al.*, 2000; Schneeweis *et al.*, 2005). Furthermore, full-length OPG possesses RANKL-and TRAIL-independent biological properties, mainly related to the promotion of endothelial cell survival as well as angiogenesis and metastasis of breast cancer, ameloblastomas, and multiple myeloma (Holen *et al.*, 2002; Shipman and Croucher, 2003; Zauli *et al.*, 2009). Therefore, OPG expression in tumor cells seems to be important in aspects of hallmark of cancer, such as metastasis. However, the details of OPG expression in cancer cells are still unclear.

OPG expression was induced by IL-1β or TPA in MG-63 cells, which is consistent with the report by Mori *et al.* (2007). Several papers reported the expression of OPG in MG-63 cells via ELISA. In this paper, we examined the expression of OPG by Western blotting. In Fig. 1, 2, we also examined the OPG expression in the media from IL-1β- or TPA-treated treated MG-63 cells by Western blotting. A 110 kda band, which corresponds to the double molecular weight of 55 kda OPG, were observed in the media of IL-1β treated MG-63 cells by Western blotting (Fig. 1B). Induction of OPG by IL-1β is stronger than that by TPA.

Tgase-2 was also highly expressed in MG-63 cells by IL-1β or TPA. To our knowledge, IL-1β- or TPA-induced Tgase-2 expression was firstly observed in MG-63 cells (Fig. 1, 2), although, the expression of Tgase-2 in MG-63 cells was also reported by Heath *et al.* (2001).

MG-63 cells treatment with IL-1β resulted in the phosphorylation of c-Jun NH2-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK). Both p38 and ERK, but not JNK, were needed for IL-1β induced OPG production. In contrast, NF-κB was not essential for IL-1β induction of OPG

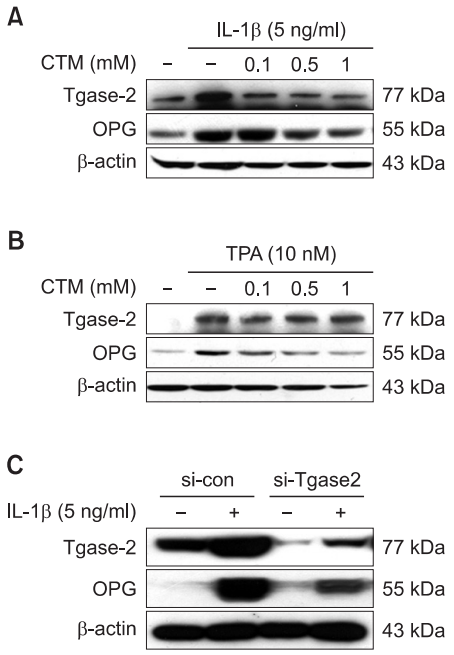

Fig. 3. Effects of CTM, and gene silencing of Tgase-2 on the OPG expression in activated-MG-63 osteosarcoma cells. (A) CTM, dose-dependently suppressed the IL-1β-induced OPG expression of MG-63 cells. MG-63 cells were treated with CTM (0.1, 0.5, 1 mM) for 1 hr. Then, cells were treated with IL-1β for 24 h. Whole-cell lysates were prepared and the protein level was subjected to 4-12% SDS-PAGE, and expression of Tgase-2 and OPG were determined by Western blotting. (B) CTM, dose-dependently suppressed the TPA -induced OPG expression of MG-63 cells. MG-63 cells were treated with CTM (0.1, 0.5, 1 mM) for 1 hr. Then, cells were treated with TPA for 16 h. (C) Involvement of Tgase-2 in activated-MG-63 cells. MG-63 cells were transfected with siRNA of Tgase-2 (50 nM) for 48 hr. The cells were then treated with IL-1β (5 ng/ml) for 24 h. Whole-cell lysates were prepared and the protein level was subjected to 4-12% SDS-PAGE, and expression of Tgase-2 and OPG were determined by Western blotting.

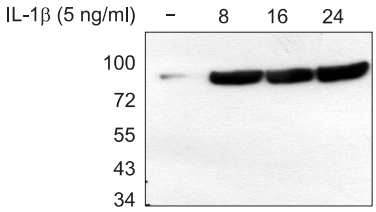

Fig. 4. Expression of N-epsilon gamma glutamyl lysine in IL-1β stimulated MG-63 osteosarcoma. MG-63 cells were treated with the control media or IL-1β (5 ng/ml) for the indicated time. Conditional media were prepared and the protein level was subjected to 4-12% SDS-PAGE, and expression of N-epsilon gamma glutamyl lysine was determined by Western blotting.

(Lambert *et al.*, 2007), although it was reported that *P. gingivalis* upregulated the expression of OPG via a NF-κB dependent pathway (Kobayashi-Sakamoto *et al.*, 2004). As such, we questioned the role of Tgase-2 in OPG expression since Tgase-2 is also induced by several inducers of OPG, such as IL-1β or TPA.

CTM, a Tgase inhibitor, suppressed the expression of OPG and Tgase-2 in IL-1β or TPA treated MG-63 cells (Fig. 2). It seemed that the inhibitory effects of CTM is strong in TPA-treated MG-63 cells since the expression of OPG by IL-1β is stronger that that by TPA (Fig. 2B).

We examined the gene silencing of Tgase-2 on the IL-1β-induced OPG expression since CTM is not a specific Tgase-2 inhibitor. Gene silencing of Tgase-2 suppressed the expression of OPG (Fig. 3C). However, the detailed effects of Tgase-2 on OPG expression are still unknown. Tgase-2 induced the upregulation and polymerization of osteopontin, which is a mineral-binding protein abundant in most mineralized tissues and pathologically calcifying tissues, including blood vessels (Speer *et al.*, 2005; Kaartinen *et al.*, 2007). Polymerized osteopontin showed enhanced biological activity, such as cell adhesion, spreading, focal contact formation, and migration (Higashikawa *et al.*, 2007). Therefore, OPG also might be a substrate and polymerized by Tgase-2. Hence, we speculated that a 110 kda band might be the result of Tgase-2's action on OPG. Thus, we tested the existence of the isopeptide bond in a 110 kda band since isopeptide bond is formed by Tgase-2. However, we did not observe the isopeptide bond in 110 kda position (Fig. 4). Therefore, a 110 kda band might be a homodimer form of OPG and disulfide linkage might be involved.

Recently, the roles of OPG in cancer are reported in several groups. For example, OPG overexpression by breast cancer cells enhanced tumor growth, following orthotopic inoculation (Fisher *et al.*, 2006). Investigation of various human cancers demonstrated endothelial OPG expression in 59% of malignant tumors (n=512), but in contrast, OPG was absent in endothelial cells associated with benign tumors and normal tissues (n=178) (Cross *et al.*, 2006). OPG functions as a paracrine survival factor for human myeloma cells (Shipman and Croucher, 2003).

Osteosarcoma is the most common skeletal sarcoma, which appears more commonly in the second to third decades of life. Although the outcome of osteosarcoma treatment has been improved by the chemotherapy-based combination therapy, progress has been painfully slow for the past 20 years (De Toni *et al.*, 2008). Therefore, Tgase-2 expression and involvement in OPG expression in MG-63 cells might be a clue for understanding the role of OPG in osteosarcoma cancer (Fig. 1, 2).

All together, we showed that Tgase-2 is involved in OPG expression and Tgase-2 might be a new way of controlling the expression of OPG in cancer cells.

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