INTRODUCTION

Osteoprotegerin (OPG) is a secreted glycoprotein belonging to 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

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 tumor 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 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 Ca2+-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 IκB (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-
increased in osteoarthritis (OA) joint cartilage (Rosenthal et al., 1997). Tgase-2 is also expressed in hypertrophic chondrocytes (Nurminskaya et al., 2003). Tgase-2 is 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) at room temperature. Immunoactive proteins were blotted onto a nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA) at room temperature. Immunoactive proteins were detected using the WEST-ZOL (plus) Western Blot Detection System (INIRON, Gyeonggi, Korea).

Cell proliferation assay

Cell proliferation was measured using the EZ-Cytox Cell viability assay kit (Daellab service, Seoul, Korea). Briefly, 100 μl of cell suspension (5,000 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 (Multiscan, 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′-AAAGCCGAAGUGAUCGGAC-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 fraction 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 Tpase-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 cells.

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 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. Immunoreactive proteins were detected using the WEST-ZOL (plus) Western Blot Detection System (INIRON, Gyeonggi, Korea).
with control media or IL-1β 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.

**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; Schneweis 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.

**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; Schneweis 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.
Polymerized osteopontin showed enhanced biological activity stronger than that by TPA (Fig. 2B).

Blood vessels (Speer al., 2005; Kaartinen et al., 2007), although it was reported that *P. ginvialis* 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 than 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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