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Original

Low Concentration of Etoposide Induces Enhanced Osteogenesis in MG63 Cells via Pin1 Activation

Hiroki Nakashima[1,2], Madoka Yasunaga[1,2], Mizuki Yoshida[2,3], Masahiro Yamaguchi[2,3], Saki Takahashi[1,2], Hiroshi Kajiya[2,4], Sachio Tamaoki[1] and Jun Ohno[2]

[1] *Section of Orthodontics, Department of Oral Growth and Development, Fukuoka Dental College, Fukuoka, Japan*
[2] *Oral Medicine Research Center, Fukuoka Dental College, Fukuoka, Japan*
[3] *Section of Gerodontology, Department of General Dentistry, Fukuoka Dental College, Fukuoka, Japan*
[4] *Section of Geriatric Dentistry, Department of General Dentistry, Fukuoka Dental College, Fukuoka, Japan*
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Abstract: This study aimed to examine whether a low concentration of etoposide can accelerate osteogenesis via the activation of peptidyl-prolyl isomerase Pin1 in human MG63 cells using a genetic knockdown approach. MG63 cells treated with 0.1 μM etoposide for 24 h showed neither reduction in cell viability nor induction of cellular senescence; however, there was a significant increase in the percentage of nuclear staining with γH2AX as compared with that in untreated control cells, indicating that 0.1 μM etoposide induces weak DNA damage response (DDR) in the cells. Treatment with 0.1 μM etoposide accelerates osteogenesis in osteogenic induction medium (OIM)-cultured MG63 cells, demonstrating increased expression of Runx2 and Osterix and intense alkaline phosphatase (ALP) staining, as compared with cells treated without etoposide. In addition to those osteogenic markers, Pin1 expression was upregulated in the etoposide-treated cells, suggesting that the weak DDR may provide an interaction between Pin1 activation and osteogenic markers. The RNA interference-mediated silencing of Pin1 suppressed the expression of osteogenic markers and ALP staining in the OIM-cultured cells pretreated with 0.1 μM etoposide. Based on these findings, we suggest that a low concentration of etoposide induces mild DDR to activate Pin1, eventually promoting OIM-induced osteogenesis in the MG63 cells.

Key words: Etoposide, Human MG63 cells, Osteogenesis, DNA damage response, Pin1

Introduction

DNA damage, which occurs in all cells, is categorized into two types: endogenous and exogenous[1,2]. Endogenous damage, caused by the cell itself, can arise from various pathways, such as apoptosis, excision repair, oxidative damage, or depurination, while exogenous damage occurs when cells are exposed to physical damage, such as ultraviolet light and ionizing radiation, or to chemical agents, such as reactive oxygen species, intercalating agents, alkylating agents, and base analogs. These agents can cause damage to the DNA directly, indirectly, or both.

When DNA-damaging factors are present, the cellular DNA damage response (DDR) is induced to store and send genetic information, for cancer transformation, to induce cellular senescence, and for cell survival and death. DNA damage is divided into three types: double-stranded DNA breaks (DSBs), single-stranded DNA breaks, and base–nucleotide modifications[3]. The DDR is initiated by damage detection, followed by signal transmission to effectors, ultimately resulting in a number of cellular responses, such as induction of cell-cycle arrest, lesion repair, or, in the case of non-repairable damage, the activation of the cell death program[4]. Depending on the cell type and the severity and extent of the DNA damage, the activated DDR can elicit different cellular responses.

In response to DNA damage, the DDR is initiated by phosphatidylinositol 3-kinase-like kinases, such as ataxia telangiectasia mutated (ATM), which responds primarily to DSBs. Once activated, ATM phosphorylates downstream checkpoint kinases, which in turn phosphorylate and activate p53 to affect cell progression[5]. Among many cellular alterations in DNA damage, the DDR to mild DNA damage normally results in repair with or without cell-cycle arrest, whereas the occurrence of severe and irreparable injury shifts the cellular response toward the induction of senescence or cell death programs, such as apoptosis, autophagy, and necrosis[4,6]. ATM activated by DNA damage triggers cell-cycle checkpoints, apoptosis, and repair. Furthermore, a recent study speculated that damage to the DNA via activated ATM may be involved in osteoblastic function, bone formation, and bone remodeling, without a cell-autonomous effect on osteoblast and bone resorption[7]. However, the mechanism of DNA damage-induced osteogenesis remains unclear.

Peptidyl-prolyl isomerase Pin1 plays an important role in regulating cellular homeostasis by isomerizing the propel bond preceded by a phosphorylated Ser or Thr residue[8]. As the isomerization by Pin1 regulates the biological function of several target proteins, such as cell-cycle regulators, proto-oncogenes, tumor suppressors, and transcription factors[9], Pin1 tightly controls cell-cycle progression and cell division and in turn also regulates cellular senescence[2], suggesting that Pin1 may also regulate the DDR. Furthermore, recent reports have shown that the interaction of Pin1 with runt-related transcription factor 2 (Runx2), the major transcription factor for osteoblast differentiation, is critical for bone development[10,11]. In this study, we use a genetic knockdown approach to investigate whether etoposide-induced DDR can enhance os-

Correspondence to: Dr. Jun Ohno, 2-15-1 Tamura, Sawara-ku, Fukuoka, Fukuoka 8140193, Japan; Tel: +81928010411 (Ext 1684); Fax: +81928014909; E-mail: johno@college.fdcnet.ac.jp

teogenic differentiation of MG63 cells via the Pin1 signaling pathway.

Materials and Methods

Cell culture

Human preosteoblast MG63 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Fujifilm Wako Pure Chemical Co, Osaka, Japan) with 10% (v/v) fetal bovine serum (FBS; HyClone Laboratories Inc, South Logan, UT, USA) and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific Inc, Rockford, IL, USA). All cultures were maintained at 37°C in a humidified incubator and with 5% CO_2. Once the cells reached subconfluence, they were treated for 24 h with medium containing various concentrations of etoposide (0, 0.1, 1, and 10 μM). After stimulation with etoposide, the cells were cultured in DMEM with FBS supplemented with osteogenic induction medium (OIM) containing ascorbic acid, dexamethasone, and β-glycerophosphate (TaKaRa Bio Inc, Tokyo, Japan) for 7 days.

Cell viability assay

MG63 cells were seeded in 96-well plates with 100 μl medium at a density of 1×10^4 cells/ml and incubated at 37°C overnight to allow the adherent cells to attach to the wells. The cultured cells were then incubated with etoposide (0–10 μM) for 24 h. Next, 10 μl Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was added to each well, and the cultured cells were incubated at 37°C for 4 h. The optical density was measured at 480 nm.

Senescence-associated β-galactosidase (SA-β-Gal) staining

We used SPiDER-βGal (Dojindo Laboratories) for SA-β-Gal staining. MG63 cells treated with or without etoposide on a 10-well glass chamber slide were fixed with 4% (w/v) paraformaldehyde for 10 min and stained with SPiDER-βGal working solution at 37°C for 30 min. To enable visualization of the nuclei, cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc, Burlingame, CA, USA).

Alkaline phosphatase (ALP) staining

After a 7-day incubation with OIM, in the presence or absence of stimulation with various concentrations of etoposide for 24 h, the MG63 cells were stained with an ALP kit (Sigma-Aldrich Co, St. Louis, MO, USA), according to the manufacturer's instructions.

Western blot analysis

The cells were lysed in cell lysis buffer (Cell Signaling Technology Inc, Danvers, MA, USA) containing a 1× protease/phosphatase inhibitor cocktail (Cell Signaling Technology). The protein content was measured using a protein assay kit (Pierce, Hercules, CA, USA). Western blot was performed using the WES automatic Western system[12] (ProteinSimple Inc, San Jose, CA, USA): 1.2 μg total protein of the samples was loaded to each lane, and 12–230 kDa separation modules were used. The peak areas of the bands were measured using Compass software (ProteinSimple).

Immunocytochemical analysis

MG63 cells were cultured on 10-well glass slides, fixed with 4% paraformaldehyde for 10 min, and washed in 0.3% Triton-X in phosphate-buffered saline (PBS) for 10 min. Cells were incubated with anti-Runx2 (1:100; Cell Signaling Technology), anti-Sp7/osterix (1:100; Abcam Inc, Cambridge, UK), anti-γH2AX (1:100; Dojindo Laboratories), anti-Pin1 (1:100; Cell Signaling Technology), and anti-β-actin (1:200; Cell Signaling Technology), at 4°C overnight. After washing with PBS, the cells were incubated with anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG conjugated with Alexa Fluor 488 or 556 (1:400; Molecular Probes, Eugene, OR, USA) at room temperature for 45 min. To visualize the nuclei, the cells were counterstained with DAPI. The percentages of positive cells were estimated under the 40× objective lens of a fluorescence microscope at five different sites of each slide glass.

Blockade of Pin1 expression

Small-interfering RNA (siRNA) for Pin1 was used to inhibit Pin1 expression. The cells were seeded in 24-well plates at a density of 5×10^4 cells/well and were transfected with siRNA for Pin1 (5′-GAAGAU CACCCGGACCAAG-3′) and a negative control siRNA (5′-AACGU ACGCGGAAUACAACGA-3′), which were purchased from Hokkaido System Science Co., Ltd. (Sapporo, Japan)[13]. siRNA was introduced into the cells using HilyMax (Dojindo Laboratories). The cells were stimulated with or without etoposide 24 h thereafter. After treatment with or without etoposide for 24 h, those cells were cultured in OIM for 7 days to perform the assay. All siRNA experiments were performed in triplicate.

Statistical analysis

All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics. The analysis was performed with one-way analysis of variance with Tukey's multiple comparison test or paired Student's *t* test to determine the statistical differences among the samples. Data are presented as the mean ± standard deviation (SD), and p values <0.05 were considered to be statistically significant.

Results

Treatment with 0.1 μM etoposide does not significantly reduce cell viability

MG63 cells were treated with 0 (as a control), 0.1, 1.0, and 10 μM etoposide for 24 h to assess the cell viability of each group using the CCK-8 assay. As shown in Fig. 1, 0.1 μM treatment showed no significant decrease in cell viability as compared with the control group (p = 0.67), whereas the other groups remarkably reduced the viability of MG63 cells (control and 0.1 μM vs 1.0 μM, p = 0.032; control and 0.1 μM vs 10 μM, p = 0.002) in a dose-dependent manner.

Low concentration of etoposide treatment can induce DNA damage but not cellular senescence

Etoposide, a topoisomerase II inhibitor, induces DNA damage. During DNA double-strand breaks by stimulus, γH2AX is produced by phosphorylation of H2AX. To examine whether etoposide can induce DNA damage in MG63 cells, we performed immunocytochemical staining of γH2AX (Fig. 2A). Most of the untreated MG63 cells (5% ± 2.2%) showed an absence of nuclear γH2AX staining. Pannuclear fluorescence patterns of γH2AX were obtained in the cells treated with 0.1 (25.4% ± 4.4%), 1.0 (40.4% ± 4.2%), and 10 μM (98.8% ± 1.8%) etoposide (Fig. 2B). The percentages of pannuclear staining were significantly higher in cells treated with 0.1 μM etoposide than in control cells ($p < 0.001$). The increased concentration led to a higher percentage of positive cells. These findings reveal that etoposide-induced H2AX phosphorylation

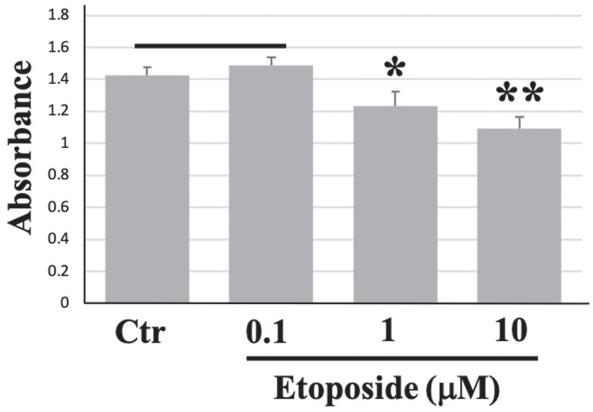

Figure 1. Effect of etoposide on the cell viability by Cell Counting Kit-8 (CCK-8) assay (n = 3). The cell viability of MG63 cells treated with 0 (control) to 10 μM etoposide for 24 h using the CCK-8 assay. There was no significant between-group difference of the gray columns joined to the horizontal bar. *Significantly different at $p < 0.05$ as compared with the group treated with 1 μM etoposide. **Significantly different at $p < 0.01$ as compared with the group treated with 10 μM etoposide.

might be a result of DNA damage in MG63 cells.

Etoposide can induce both senescence and apoptosis[5]. We next investigated whether etoposide treatment can induce cellular senescence in MG63 cells by SA-β-Gal staining (Fig. 2C). Untreated control cells did not show any cytoplasmic reaction with the staining. Senescent cells induced by etoposide treatment showed cytoplasmic staining. There were no significant differences in the percentage of SA-β-Gal-positive cells between the control and 0.1 μM groups (control, 5.6 ± 3.7 vs 0.1 μM, 16.4 ± 11.9; $p = 0.21$; Fig. 2D). Both the 1 and 10 μM groups showed higher percentages of positive cells as compared with control and 0.1 μM groups ($p < 0.001$). From these findings, we suggest that MG63 cells treated with the minimal dose of etoposide (0.1 μM) can lead to a pre-senescent status by DNA damages, although DNA-damaged senescence is induced in the cells treated with 1 μM or greater etoposide.

Treatment with 0.1 μM etoposide accelerates osteogenesis in OIM-treated MG63 cells

To investigate whether a low concentration of etoposide treatment can promote osteogenesis in MG63 cells cultured in the presence of OIM, we first analyzed the protein expression of osteogenic markers,

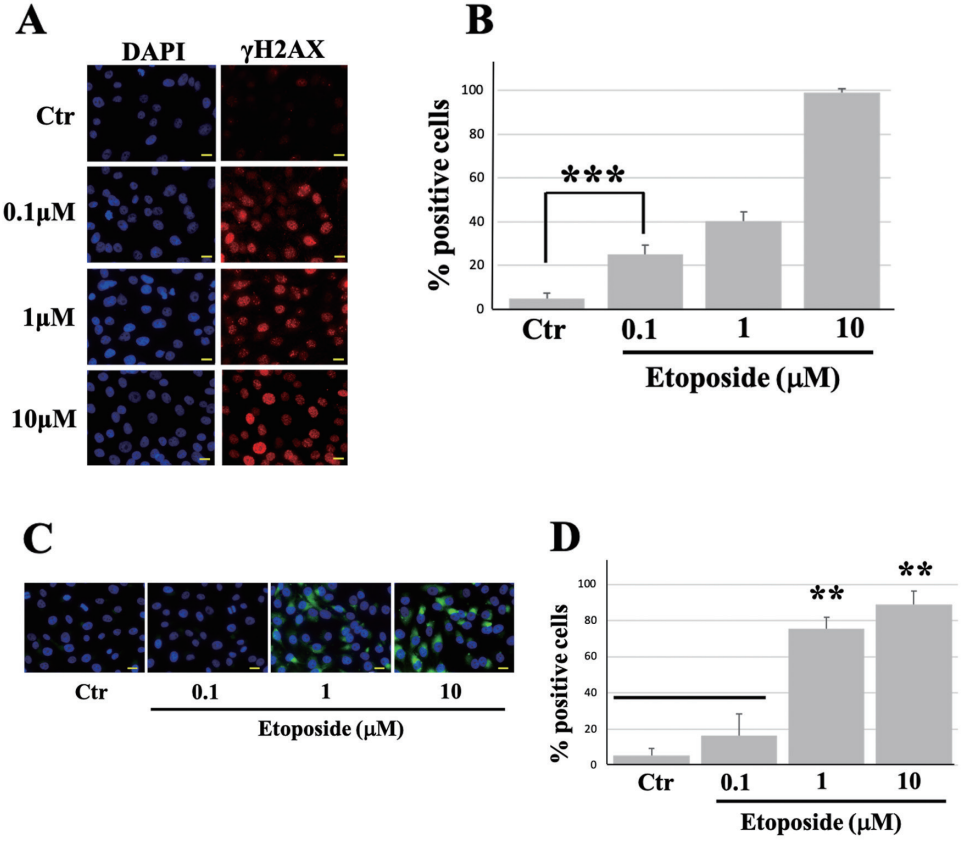

Figure 2. Effect of etoposide on DNA damage and cellular senescence. (A) DNA damage of MG63 cells treated with 0 (control) to 10 μM etoposide for 24 h by immunocytochemical detection of γH2AX. Immunofluorescence staining was used to assess the nuclear reaction of γH2AX. Cells were stained with anti- γH2AX antibody (red) and DAPI (blue). Bars = 20 μm. (B) Semiquantification of the percentages of nuclear-positive cells stained with anti-γH2AX antibody. ***Significantly different at $p < 0.001$ as compared with the control group (n = 3). (C) A fluorescence image of MG63 cells stained with SPiDER-βGal (green) after treatment with various concentrations of etoposide (24 h) or untreated (control). Nuclei were stained with DAPI (blue). Bars = 20 μm. (D) Quantitated data of the percentage of positive cells stained with the SPiDER-βGal as mean ± SD (n = 3). There was no significant difference between groups of the gray columns joined to the horizontal bar. **Significantly different at $p < 0.01$ as compared with control and 0.1 μM etoposide treatment.

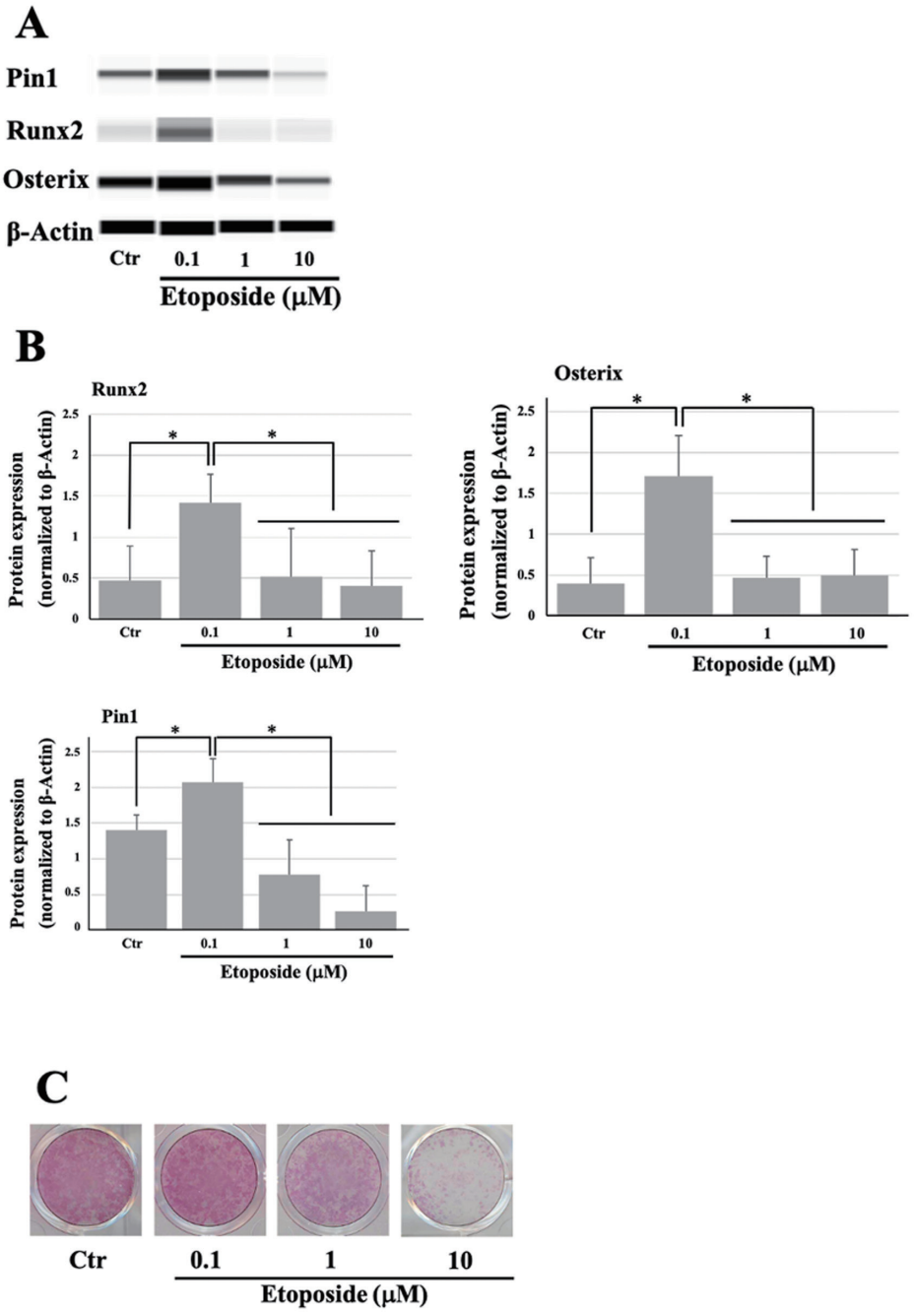

Figure 3. Enhancement of osteogenesis in OIM-treated MG63 cells after pretreatment with a low concentration of etoposide. (A) Protein expression of Pin1, Runx2, Osterix, and β-actin in OIM-treated MG63 cells after pretreatment with or without various concentrations (0.1, 1, and 10 μM) of etoposide, via Western blot. (B) Quantification graphs of proteins performed using the Wes system. Values shown were the fold increase normalized to those of β-actin. Similar results were obtained in three independent experiments. *Significantly different at $p < 0.05$ as compared with control or 1, 10 μM etoposide treatment and 0.1 μM etoposide treatment. (C) Representative images of ALP activity in OIM-treated cells after pretreatment with or without various concentrations (0.1, 1, and 10 μM) of etoposide.

such as Runx2, Osterix, and Pin1, in the OIM-exposed cells after pretreatment with or without etoposide using Western blot (Fig. 3A, B). Expression of those markers was significantly upregulated in cells treated with a combination of 0.1 μM etoposide pretreatment and OIM as compared with those in the OIM alone group and those receiving a combination of 1 and 10 μM etoposide pretreatment and the OIM groups.

We next performed ALP staining intensity on the OIM-treated cells after stimulation with or without etoposide because the histochemical detection of ALP activity is considered an early marker of osteogenesis. The intensity of ALP staining was significantly enhanced in cells pretreated with 0.1 μM etoposide as compared with cells not pretreated and cells pretreated with other concentrations (1, 10 μM) of etoposide (Fig. 3C). These results indicate that pretreatment with 0.1 μM etoposide can enhance OIM-induced osteogenesis in the MG63 cells.

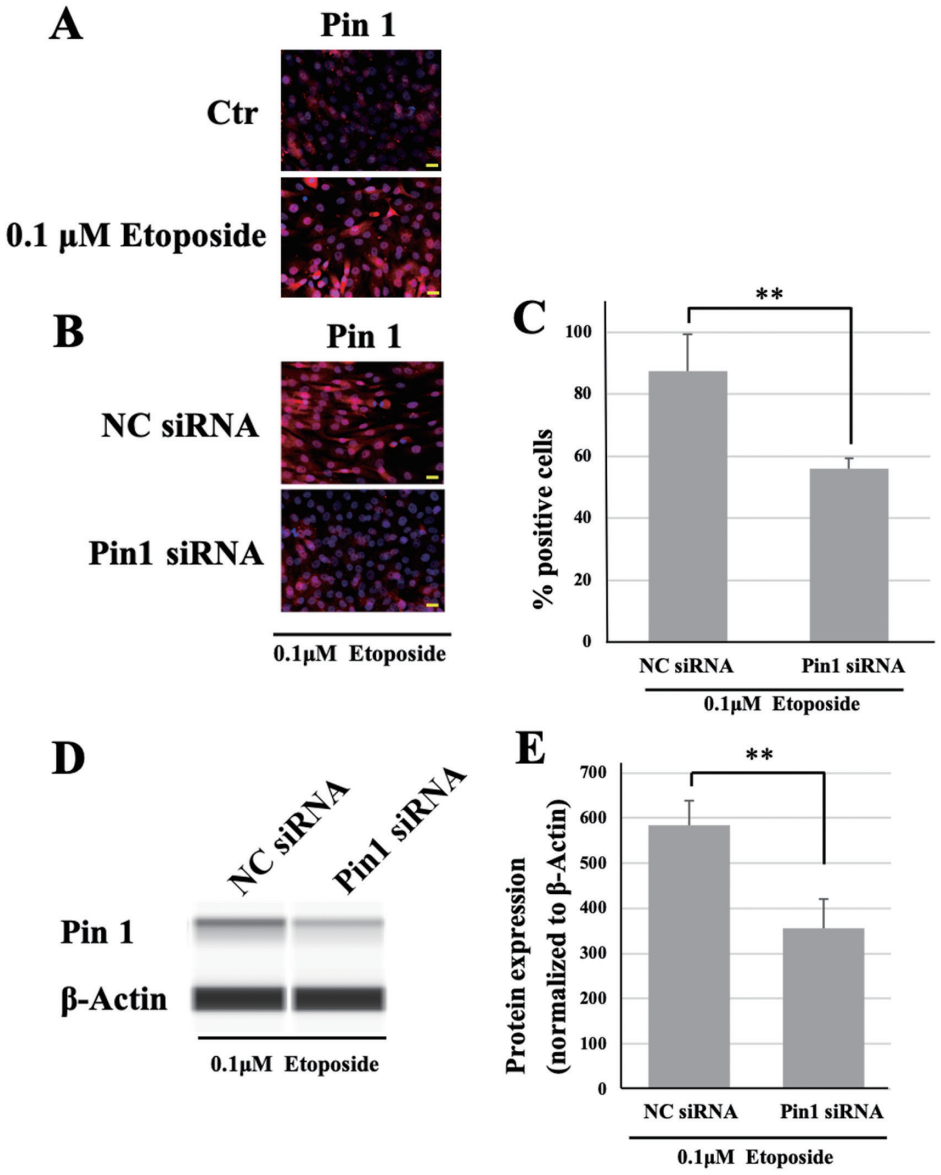

Figure 4. Inhibition of 0.1 μM etoposide-induced nuclear translocation of Pin1 by the blockade of Pin1 signaling in the MG63 cells. (A) Representative immunofluorescence images of the nuclear expression of Pin1 in MG63 treated with or without 0.1 μM etoposide for 24 h. Cells were stained with anti-Pin1 (red) and DAPI (blue). Ctr, control (untreated) cells. Bars = 20 μm. (B–E) MG63 cells were treated with 0.1 μ M etoposide in the presence of a Pin1 siRNA or NC siRNA for 24 h. (B) Changes in the nuclear translocation of Pin1 by immunofluorescence staining. Bars = 20 μm. (C) A quantification graph of the percentage of Pin1-positive cells as mean ± SD (n = 3). **Significantly different at $p < 0.01$ as compared with NC siRNA and Pin1 siRNA. (D) Expression of Pin1 protein by Western blotting. (E) A quantification graph of protein was performed using the Wes system. Values shown were the fold increase normalized to those of β-actin. Similar results were obtained in three independent experiments. **Significantly different at $p < 0.01$ as compared with NC siRNA and Pin1 siRNA. NC siRNA, negative control siRNA.

Silence of Pin1 inhibits its nuclear translocation

Nuclear localization and accumulation of Pin1 are necessary for its activity. To examine whether 0.1 μM etoposide treatment can induce the nuclear activity of Pin1, we transfected Pin1-specific siRNA in MG63 cells. Via immunofluorescence staining, we found that the nuclear expression of Pin1 was increased in cells treated with 0.1 μM etoposide for 24 h as compared with that in untreated (control) cells (Fig. 4A). After Pin1 was silenced using siRNA, the nuclear translocation and protein expression of Pin1 were significantly decreased as compared with those in the cells transfected by the negative control siRNA (Fig. 4B-E).

Attenuation of Pin1 expression reduces etoposide-accelerated osteogenesis in the MG63 cells

The effect of Pin1 silencing on etoposide-accelerated osteogenesis was examined in OIM-cultured MG63 cells pretreated with 0.1 μM etoposide in the presence of Pin1 siRNA or negative control siRNA by immunofluorescence staining and Western blot analysis and ALP staining (Fig. 5). The results of both immunofluorescence and Western blot assays showed that as compared with transfection with a negative control siRNA, transfection with Pin1-specific siRNA significantly decreased the expression of Runx2 and Osterix (Fig. 5A-F). Furthermore,

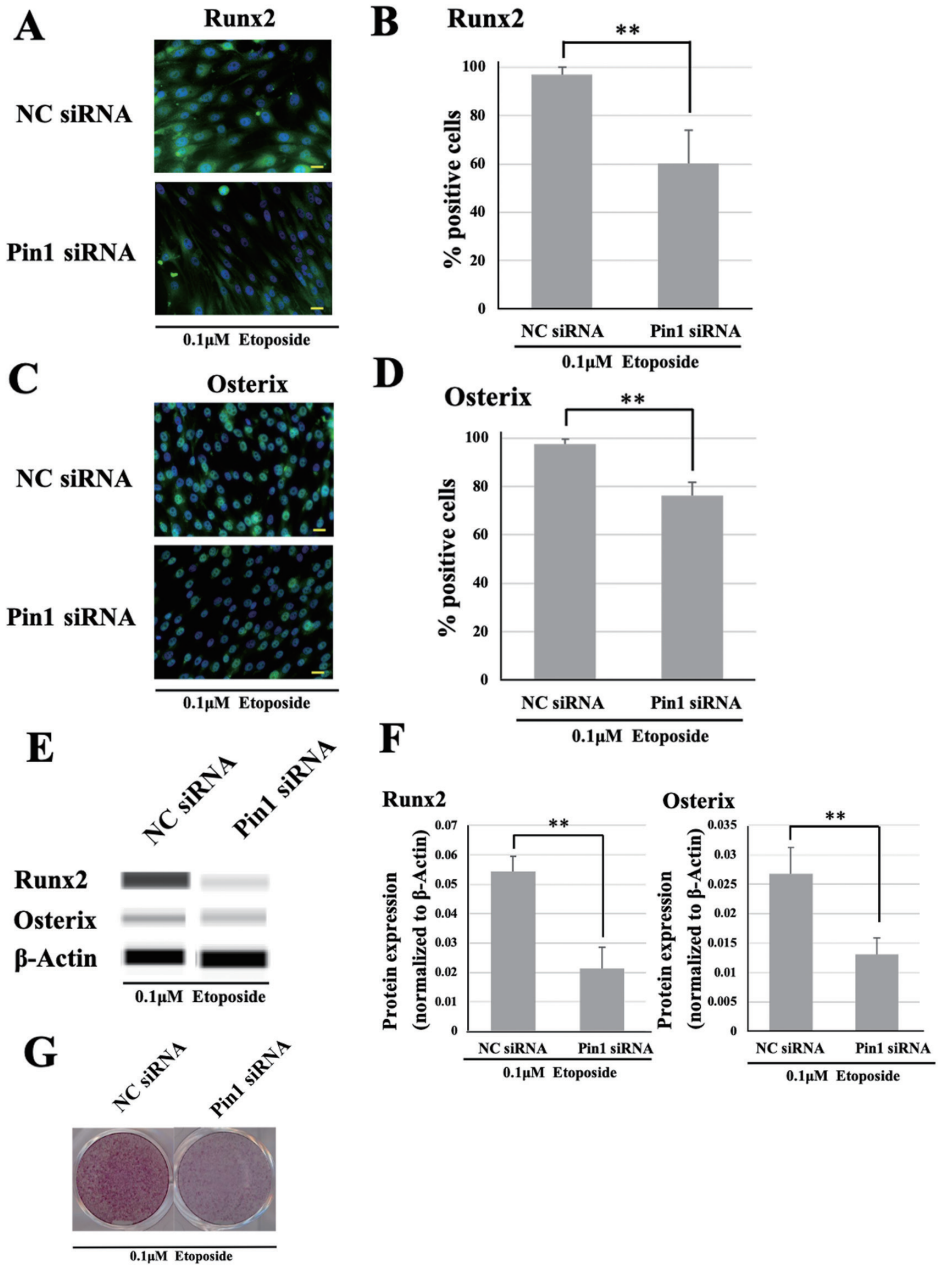

Figure 5. The inhibition of etoposide-accelerated osteogenesis via the transfection of Pin1 siRNA in MG63 cells. OIM-cultured MG63 cells were pretreated with 0.1 μM etoposide in the presence of negative control siRNA (NC siRNA) and Pin1 siRNA. (A) Representative images of Runx2 expression by immunofluorescence staining. Immunocytochemical reactivity and DAPI-stained nuclei showed green and blue, respectively. Bars = 20 μm. (B) Semiquantification of the percentage of positive cells stained with anti-Runx2 antibody as mean ± SD (n = 3). **Significantly different at $p < 0.01$ as compared with NC siRNA and Pin1 siRNA. (C) Representative images of Osterix (OSX) expression by immunofluorescence staining. Immunocytochemical reactivity and DAPI-stained nuclei are showed green and blue, respectively. Bars = 20 μm. (D) Semiquantification of the percentage of positive cells stained with anti-OSX antibody as mean ± SD (n = 3). **Significantly different at $p < 0.01$ as compared with NC siRNA and Pin1 siRNA. (E) Western blot assay of Runx2, Osterix, and β-actin. (F) Quantification graphs of protein were performed using the Wes system; values shown were the fold increase normalized to those of β-actin. Similar results were obtained in three independent experiments. **Significantly different at $p < 0.01$ as compared with NC siRNA and Pin1 siRNA. (G) Representative images of ALP activity. NC siRNA, negative control siRNA.

cells transfected with Pin1 siRNA showed a decreased intensity of ALP staining as compared with negative control siRNA (Fig. 5G). These findings indicate that Pin1 silencing attenuates the osteogenesis in the MG63 cells accelerated by 0.1 μM etoposide.

Discussion

The findings of a previous study suggested that, depending on its severity, the DDR may be involved in osteogenesis[7]. In this study, we provide three lines of evidence indicating that a low concentration of etoposide facilitates OIM-induced osteogenesis in MG63 cells via the

activation of the Pin1 signaling pathway by induction of mild DNA damage. First, DNA damage in the MG63 cells is induced by 0.1 μM etoposide, but this concentration provides neither decrease in cell viability nor induction of cellular senescence. Second, mild DNA damage induced by 0.1 μM etoposide can accelerate the OIM-induced osteogenesis in the cells, which indicates the upregulated expression of osteogenesis-related factors and increased intensity of ALP activity. Third, transfection of Pin1 siRNA into MG63 cells reveals a significant suppression of etoposide-accelerated osteogenesis.

The findings from our cell viability examination indicate that DNA damage and cellular senescence provide unique characteristics of MG63 cells treated with 0.1 μM etoposide. The intense fluorescence of γH2AX staining was observed at this concentration in the nuclei of MG63 cells, whereas neither decreased absorbance rates of CCK-8 assay nor upregulated intensity of cytoplasmic SA-β-Gal staining was observed in these cells. Treatment with 0.1 μM etoposide may promote mild DNA damage in the MG63 cells. Etoposide induces DNA damage with DSBs to cells by interacting with the nuclear enzyme topoisomerase II and can lead to cellular senescence and apoptosis through concentration dependence[14]. The etoposide concentration used in this study provides a weak DDR showing the pre-senescent status without inducing cell-cycle arrest. Based on these findings, we speculate that in this condition, cells treated with etoposide can maintain the activity of DNA repair and continue their function.

The most striking finding is that treatment with 0.1 μM etoposide led to the enhancement of OIM-induced osteogenesis in the MG63 cells, suggesting that accelerated osteogenesis may be involved in one of the weak DDR events included in the 0.1 μM etoposide treatment. Since the differentiation process of the cells is affected by DNA damage without induction of cell-cycle arrest[7], we postulate that a weak DDR can activate the potential for osteogenic capability in the MG63 cells. Our results showed that etoposide induced a weak DDR, which represents the increased expression of the osteogenic master regulators Runx2 and Osterix[15-17], in the OIM-cultured MG63 cells. Although the precise mechanisms of DNA damage-induced osteogenesis remain unclear, we suggest that the weak DDR activates ATM to eventually activate the components in the OIM mixture and promote the phosphorylation of Runx2[18]. Recent studies have also focused on the relationship between DNA damage and osteogenesis[7,19].

In this study, we demonstrated that a weak DDR induced the increased expression of Pin1 in the OIM-cultured cells. Activated Pin1 resulting in the weak DDR can contribute to the regulation of the cell-cycle progression, cell division, and cell differentiation[8,9]. Furthermore, Pin1 plays a pivotal role in DNA damage-triggered cell fate decisions, resulting in DNA repair or induction of apoptosis or cellular senescence[20]. In recent studies, researchers postulated that activated Pin1 is closely related to enhanced osteogenic activity in various cells[10,21,22]. In accordance with these findings, we suggest that the Pin1 expression induced by DDR may mediate accelerated OIM-induced osteogenesis of MG63 cells.

Gene silencing of Pin1 demonstrated that Pin1 plays a critical role in the enhanced osteogenesis of MG63 cells treated with 0.1 μM etoposide. The results of the immunofluorescence assay demonstrated that the percentage of nuclear Pin1-positive cells was remarkably decreased in 0.1 μM treated cells in the presence of Pin1 siRNA than in the presence of negative control siRNA, indicating that the gene silencing of Pin1 suppressed the activity of Pin1 in the etoposide-treated cells. After the blockade of Pin1 expression by siRNA, the etoposide- and OIM-treated MG63 cells demonstrated a lower expression of Runx2 and Osterix by both Western blotting and immunofluorescence assays. Furthermore, those cells showed weak intensity of ALP staining as compared with cells in the presence of negative control siRNA. Activated Pin1 is a critical regulator of osteogenic factors, such as Runx2 and Osterix, known as the most famous transcription factors for osteoblast differentiation, both in vitro and in vivo[10,22,23]. In particular, Pin1 enhanced the differentiation of osteoblasts, by which Pin1 interacted and affected the protein stability and transcriptional activity of Runx2[21]. The authors of a recent study demonstrated that Pin1 could be a critical target for modulating Wnt/β-catenin-mediated osteogenesis[11]. From these findings, we suggest that activated Pin1 may promote etoposide-induced osteogenesis in OIM-cultured MG63 cells via the direct interaction of Pin1 with the master transcription factors for bone formation.

This study has some possible limitations, especially the lack of direct evidence regarding which osteogenic pathway activated by etoposide-induced DNA damage can be regulated by Pin1. Our previous findings indicated that OIM-induced osteogenesis is related to both the Smad-dependent bone morphogenetic protein and Wnt/β-catenin pathways[24,25]. A recent study proposed that Pin1 activation promotes Wnt3a-induced osteoblast differentiation[11]. Therefore, further studies are warranted to examine the involvement of a pathway responsible for the osteogenesis accelerated by etoposide-induced DNA damage.

In conclusion, our findings provide additional information about the effect of mild DNA damage on the osteogenic promotion in OIM-cultured MG63 cells. We demonstrated that a low concentration of etoposide induces a weak DDR that activates Pin1, leading to the eventual promotion of OIM-induced osteogenesis in MG63 cells.

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Conflict of Interest

The authors declare no conflicts of interest.

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