Original

Cisplatin-Induced Sonic Hedgehog Signaling Mediates Epithelial-Mesenchymal Transition in Hertwig’s Epithelial Root Sheath Cells

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Abstract: In this study, we determined whether cisplatin can induce epithelial-mesenchymal transition (EMT) via the activation of Sonic hedgehog (Shh) or glioma-associated antigen-1 (Gli1) signaling pathway in mouse Hertwig’s epithelial root sheath (HERS) cells using a genetic knockdown approach. HERS cells treated with a low concentration of cisplatin (0.5 µM) for 24 h showed no reduction in the cell viability; however, there was a significant increase in the percentages of nuclear staining with γH2AX as compared to that with untreated control cells, indicating that 0.5 µM cisplatin induces DNA damage. Further, 0.5-µM cisplatin-treated cells provided an induction of EMT, showing decreased and increased expression of epithelial and mesenchymal markers, respectively. Enhancement in the EMT activity in cisplatin-treated HERS cells was correlated with increased expression of Shh and accelerated translocation and accumulation of Gli1 expression into the nucleus. The RNA interference-mediated silencing of Gli1 suppressed the acceleration of EMT in cisplatin-treated HERS cells; this was confirmed by no down-regulation or up-regulation in the expression of E-cadherin and vimentin, respectively, along with no increased expression of Snail expression. These findings suggest that the activation of Shh/Gli1 signaling pathway may be required for the enhancement of EMT in cisplatin-treated HERS cells.

Key words: Hertwig’s epithelial root sheath (HERS), Epithelial-mesenchymal transition (EMT), Cisplatin, Sonic hedgehog, Glioma-associated antigen-1, E-cadherin, Vimentin, Snail

Introduction

Chemotherapeutic drugs are widely used and are effective in the treatment of pediatric cancers. Among many chemotherapeutic drugs, cisplatin, one of major platinum compounds, currently serve as the first line chemotherapy in the treatment of pediatric cancers, such as neuroblastoma, germ-cell tumors, osteosarcoma, retinoblastoma, hepatoblastoma, brain tumors, and relapsed and refractory lymphomas. Cisplatin forms DNA-protein cross-links, causing breakage of DNA strands in cancer cells, to exert cytotoxic effects and trigger apoptosis in these cells. Chemotherapy-induced DNA damage may provide some palliation; however, it is often associated with substantial treatment-related toxic effects.

Recent strong evidence supports an association between chemotherapy and dental anomalies. In particular, children who receive chemotherapy before the age of 5 y may have a high risk of extensive damage to their dentition, indicated by the development of conditions, such as hypodontia, microdontia, enamel hypoplasia, dental caries, and atypical crown or root formation. In cases of arrested root development, short V-shaped roots and early closure of the apex are relatively common findings. Root formation causes an overall increase in the tooth length to accompany intraosseous eruption during the process of emergence. Therefore, atypical root formation may cause disturbances in tooth eruption that severely impact the oral health quality of life in children receiving long-term chemotherapy.

Root and cementum formation are controlled by Hertwig’s epithelial root sheath (HERS). The HERS, originating from an elongated apical region of the enamel organ, is composed of a bilayer epithelial structure between the dental papilla and dental follicle; it grows apically and guides root formation, resulting in the determination of the size, shape, and number of the tooth’s root. Among the fate of HERS nearing completion of root formation, an induction of epithelial-mesenchymal transition (EMT) is responsible for participation in the cementum and periodontal ligament formation. Our recent report suggests that impaired root formation induced by chemotherapy may result in early closure of apex via induced EMT of HERS cells.

The EMT provides depolarization of the epithelial cells; loss of their cell-cell contacts; and gain of an elongated, fibroblast-like morphology. Functional implications of EMT include enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and substantially increased production of extracellular matrix components. In vitro ex-
periments, cells treated with transforming growth factor β1 (TGF-β1) can show the induction of marked EMT characteristics. A previous study has demonstrated that TGF-β can induce EMT in HERS cell. The EMT process is regulated in part by Sonic hedgehog (Shh) signaling that corresponds to several biological processes in normal embryonic development and adult tissue homeostasis. Once Shh binds to Patched (Pch), an inhibition of a signal transducer Smoothened (Smo) is relieved, leading to the translocation of Gli1 proteins into the nucleus and the activation of Shh genes expression. Activation of the Shh signaling pathway, including nuclear translocation of Gli1 can provide increased expression of snail, a major target of the Shh signaling pathway, as well as regulate EMT and fibroblast motility. Although few studies have discussed the possibility of chemotherapy-induced EMT whether cisplatin induces EMT in HERS cells remains unclear.

To the best of our knowledge, there has been no systematic study of the possible mechanisms of cisplatin that may induce EMT in HERS cells. Our previous study suggested that chemotherapy-induced EMT may provide the impaired root formation via an early closure of the apex. In this study, we examined whether cisplatin could trigger EMT in HERS cells via the Shh/Gli1 signaling pathway.

**Materials and Methods**

**Cells**

The HERS cell line, HERS01a, was previously established. HERS01a cells were maintained in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) supplemented with B12 ((Invitrogen, Carlsbad, CA, USA), fibroblast growth factor-2 (FGF2, 10 ng/ml, Fibroblast Spray, Kakei Pharmaceutical Co., Ltd., Tokyo, Japan), epidermal growth factor (EGF, 10 ng/ml, Pepro Tech, Rocky Hill, NJ, USA), and penicillin-streptomycin (100 U/ml) in a humidified atmosphere of 5% CO₂ and 37°C. Upon reaching subconfluence, the cells were treated with medium containing various concentration of cisplatin (0, 0.5, 1.0, 5.0, and 10 µM) for 24 h. Furthermore, we also examined the effect of TGF-β on the induction of EMT in the HERS cells. The cells were stimulated with medium containing 10 ng/ml TGF-β for 24 h.

**Cell viability assay**

HERS cells were seeded in 96-well plates with 100 µl of medium at a density of 1 × 10⁶ cells/ml; they were incubated at 37°C overnight to allow the adherent cells to attach to the wells. The cultured cells were then incubated with cisplatin (0–10 µM) for 24 h. Thereafter, 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. After incubation, the amount of formazan dye generated via cellular dehydrogenase activity was measured by absorbance at 480 nm as the detection wavelength and at 650 nm as the reference wavelength using a plate reader (Multiskan JX, Labsystems Oy, Helsinki, Finland).

**Western blot analysis**

The cells were lysed in cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) containing a 1x Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology). The protein content was measured using a protein assay kit (Pierce, Hercules, CA, USA). Protein samples, with a protein marker (Precision Plus Protein Western C Standard; Bio-Rad Laboratories, Inc., Richmond, CA, USA), were separated on 12% Mini-Protein TGX gels (Bio-Rad) for 30 min at 200 V. The separated gels were then transferred to a polyvinylidene fluoride membrane for 7 min with the Trans-Blot Turbo Transfer system (Bio-Rad) using Trans-Blot Transfer Packs. Western blots were processed on the iBind Western System (Life Technologies, Carlsbad, CA, USA) with anti-b-actin (Bio-Rad), anti-E-Cadherin (1:1,000; Cell Signaling Technology), anti-CK14 (1:1,000; Cell Signaling Technology), anti-vimentin (1:1,000; Cell Signaling Technology), anti-N-cadherin (1:1,000; Abcam, Cambridge, UK), sonic hedgehog (Shh, 1:1,000; Cell Signaling Technology), and horseradish peroxidase-conjugated anti-rabbit or -mouse secondary antibodies (Bio-Rad). The protein bands were developed with an enhanced chemiluminescence system (SignalFire Plus ECL Reagent, Cell Signaling Technology). The band density was quantified using the NIH ImageJ software.

**Immunocytochemical analysis**

The HERS cells were cultured on the 10-well glass slides and fixed with 4% paraformaldehyde for 10 min and washed in 0.3% Triton-X in phosphate buffered saline (PBS) for 10 min. The cells were then incubated with anti-E-cadherin (1:200; Cell Signaling Technology), anti-vimentin (1:200; Cell Signaling Technology), anti-glioma-associated antigen-1 (Gli1, 1:100; Cell Signaling Technology), anti-snaill (1:100; Cell Signaling Technology), and anti-γH2AX (1:100; Dojindo Laboratories), at 4°C overnight. After washing with PBS, the cells were incubated with anti-rabbit IgG or anti-mouse IgG conjugated with Alexa Fluor 488 or 556 (1:400; Molecular Probe, Eugene, OR, USA) at room temperature for 45 min. In order to visualize the nuclei, the cells were counterstained with 4, 6-diamidino-2-phenylindole (DAPI); Vector Laboratories, Inc., Burlingame, CA, USA). The percentages of positive cells were estimated under the ×40 objective lens of a fluorescence microscope at 5 different sites of each slide glass.

**Blockade of the Shh/Gli1 signaling pathway**

Small-interfering RNA (siRNA) for Gli1 was used to inhibit the Shh/Gli1 signaling pathway. The HERS01a cells were seeded in 24-well plates at a density of 5 × 10⁴ cells/well and were transfected with siRNA for Gli1 (5’-AACUCCACAGGCAUACAGGAU-3’) and a negative control siRNA (NC siRNA: 5’-AAGGUACCGGA AUACAACGA-3’) were purchased from Hokkaido System Science Co., Ltd. (Sapporo, Japan). siRNA was introduced into the cells using HilyMax (Dojindo Laboratories). Twenty-four hours thereafter, the cells were stimulated with or without cisplatin. After treatment with or without cisplatin for 24 h, those cells were stained with antibodies for downstream target factors in the Shh/Gli1 signaling pathway. All the siRNA experiments were performed in triplicate.

**Statistical analyses**

All the 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 the R commander designed to add statistical functions frequently used in biostatistics. The analyses were performed using one-way analysis of variance and Tukey multiple comparison test to determine the statistical differences among the samples. Data are presented as the mean ± standard deviation (SD) values, and p values < 0.05 were considered to indicate statistical significance.

**Results**

**Low concentration of cisplatin treatment does not decrease the cell viability**

Cell viability was detected using the CCK-8 assay. The HERS cells
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were treated with 0 (as a control), 0.5, 1.0, 5.0, and 10 µM cisplatin for 24 h to estimate the cell viability. As shown in Fig. 1, low-concentration treatment groups (0.5 and 1.0 µM) showed no significant reduction in the cell viability as compared to the control group (p > 0.2), while the other groups (5.0 µM and 10 µM) notably reduced the viability of the HERS cells (p < 0.001) in a dose-dependent manner.

Low concentration of cisplatin can induce DNA damage in HERS cells

Cisplatin kills cancer cells by damaging their DNA. During DNA damage by stimulus, γH2AX is produced via the phosphorylation of H2AX. We performed immunocytochemical detection of nuclear γH2AX to determine whether cisplatin can induce DNA damage in the HERS cells (Fig. 2A). Most untreated HERS cells showed only few positive cells (14.5% ± 2.8%). Promotion of nuclear γH2AX staining was gradually increased in the HERS cells treated with 0.5 µM and 1.0 µM cisplatin (43.2% ± 12.0% and 57.2% ± 5.4%, respectively). There was a significant difference in the proportion of nuclear staining between untreated (Ctr) and 0.5-µM cisplatin-treated cells (p = 0.012) (Fig. 2B). These findings indicate that 0.5 µM cisplatin treatment does not reduce the cell viability significantly; however, this concentration of cisplatin can induce DNA damage in the HERS cells. Thus, we selected this concentration as the effective concentration for performing further research.

Low concentration of cisplatin can induce EMT in the HERS cells

In order to investigate whether cisplatin treatment induces EMT in the HERS cells, we first examined that EMT of HERS cells was induced via stimulation of TGF-β, known as an EMT inducer. After stimulation by TGF-β, the cells acquired an elongated and mesenchymal-like morphology with loss of cell-cell contact, while the control cells remained a cobblestone morphology as epithelial characteristics (Fig. 3A). Western blot analysis showed that the protein level of both, E-cadherin and CK14 was decreased. In contrast, the cells treated with TGF-β showed increased expression of N-cadherin, as compared to that of the controls (Fig. 3B). Intensity of vimentin expression in TGF-β-treated cells was similar to that of the control cells (Fig. 3B).

Then, we examined whether low concentration of cisplatin (0.5 µM and 1.0 µM) could induce a morphological change characteristic of EMT. The HERS cells without cisplatin exhibited a cobblestone-epithelial morphology. However, the cells stimulated by cisplatin showed a mesenchymal-like morphology with an elongated shape and loss of cell-cell contact (Fig. 3C). In the western blot analysis, downregulated expression of epithelial markers (E-cadherin and CK14) in the cells treated with 0.5 µM cisplatin was noted. Cisplatin-treated cells showed increased expression of N-cadherin in a dose-dependent manner, as compared to the control cells (Fig. 3D). Vimentin expression was increased in the cells treated with 0.5 µM cisplatin, whereas in the 1.0 µM cisplatin-treated cells vimentin expression was suppressed (Fig. 3D). These findings indicated that cisplatin-induced EMT characteristics in the HERS cells.

Figure 1. Effect of cisplatin on cell viability. Cell viability of the HERS cells treated with 0 (Ctr, control)–10 µM cisplatin for 24 h using the Cell Counting Kit-8 assay. There was no significant difference in the groups of gray columns jointed to horizontal bar. *, significant difference at p < 0.001 compared with 5- and 10-µM cisplatin-treated groups.

Figure 2. Increased promotion of cisplatin-treated HERS cells with nuclear γH2AX staining. HERS cells treated with (0.5 and 1.0 µM) and without cisplatin were labeled for γH2AX (red). The nuclei were stained with DAPI (blue). A: Representative immunofluorescence images of nuclear γH2AX staining. Bars = 20 µm. B: Proportion (%) of HERS cells with nuclear γH2AX staining as mean ± standard deviation (SD) values (n = 3). *, **, Significant differences at p < 0.05, p < 0.001, respectively, compared with Ctr.
Figure 3. TGF-β and cisplatin induces EMT in the HERS cells. A: Representative phase contrast images of a morphological phenotype in the HERS cells treated with or without TGF-β. B: HERS cells were stimulated with TGF-β (10 ng/ml) or in complete medium for 24 h. Representative western blots using antibodies against N-cadherin, vimentin, E-cadherin, and CK14. C: Representative phase contrast images of EMT induction in HERS cells treated with or without cisplatin. D: The HERS cells were treated with cisplatin (0.5 and 1.0 µM) or in complete medium for 24 h. Representative western blots using antibodies against N-cadherin, vimentin, E-cadherin, and CK14. Ctr, control. Scale bars: 20 µm.

Figure 4. Shh/Gli1 pathway in the HERS cells treated with 0.5 µM cisplatin. A: Representative western blot using antibody against Shh. B and C: Immunofluorescence images of nuclear translocation of Gli1 (B; red) and cytoplasmic staining with snail (C; green) in HERS cells treated with or without cisplatin. Nuclei were stained with DAPI. The graphs show quantified data of the percentages of positive cells stained with anti-Gli1 and snail as mean ± standard deviation (SD) (n = 3). *, significant difference at p < 0.001 with Ctr. Ctr, control; NC siRNA, negative control siRNA. Scale bars: 20 µm.
In the HERS cells, 0.5 µM cisplatin induces enhancement of the Shh/Gli1 pathway.

We examined the expressions of Shh and Gli1 in HERS cells treated with or without 0.5 µM cisplatin to investigate whether Shh/Gli1 pathway is related to cisplatin-induced EMT. As indicated by the results of the western blotting analysis (Fig. 4A), the cells exposed to 0.5 µM cisplatin for 24 h showed increased Shh expression as compared to that in the control cells. The nuclear translocation of Gli1 is a key inducer of EMT. Then, we examined the immunocytochemical detection of Gli1 in HERS cells treated with or without 0.5 µM cisplatin. The nuclear localization and accumulation of Gli1 was observed in HERS cells treated with 0.5 µM cisplatin (Fig. 4B). The percentages of nuclear positive cells were much higher in the 0.5-µM cisplatin-treated cells than in the control cells (90.8% ± 2.2% vs. 10.9% ± 1.4%, p < 0.001; Fig. 4B). Furthermore, snail is a well-known target of the Shh/Gli1 signaling. We finally performed immunocytochemical detection of snail in HERS cells treated with or without 0.5 µM cisplatin. Cisplatin-treated cells showed cytoplasmic reaction in snail, while no staining was observed in the untreated cells (Fig. 4C). The percentages of snail-positive cells were higher in the 0.5-µM cisplatin-treated cells than in the control cells (71.8% ± 9.2% vs. 6.0% ± 4.0%, p < 0.001; Fig. 4C). These results suggest that cisplatin-induced EMT may be partially regulated by the Shh/Gli1 signaling pathway.

Suppression of Gli1 signaling reduces cisplatin-induced EMT in HERS cells

In order to examine whether Gli1 signaling mediates 0.5 µM cisplatin-induced EMT, siRNA was used for silencing Gli1. Transfection of Gli1 siRNA into the HERS cells attenuated immunocytochemical expression of Gli1 protein in these cells (Fig. 5A). The transfected cells were then treated with 0.5 µM cisplatin. Compared to transfection with a negative control siRNA, immunofluorescence images and semi-quantified graphs of the percentage of positive cells showed that transfection with Gli1-specific siRNA significantly decreased the EMT process, as demonstrated by the lower expression of snail and vimentin and the increased expression of E-cadherin (Fig. 5A, B). These findings suggest that inhibition of Shh/Gli1 signaling effectively suppressed the EMT process in vitro.
Discussion

Our previous findings suggested that the administration of anticancer drug can cause arrested root development of the molars in mice via an induced EMT-like phenomenon. Here we demonstrate that low concentration of cisplatin promotes EMT in the HERS cells via the activation of Shh/Gli1 signaling pathway by induction of DNA damage. Our conclusion is supported by the following observations: (a) Low concentration of cisplatin (0.5 µM) induces DNA damage; however, this concentration does not reduce the cell viability; (b) cisplatin-induced EMT is examined on the basis of downregulated and up-regulated expression of epithelial and mesenchymal markers, respectively; (c) the cisplatin-treated HERS cells showed increased Shh and Gli1 expressions; (d) Transfection of Gli1 siRNA into the HERS cells reveals a significant suppression of cisplatin-induced EMT.

Low concentration of cisplatin is critical for inducing EMT in the HERS cells. Our results from CCK-8 assay and γH2AX staining showed that 0.5 µM cisplatin treatment showed no reduction of the cell viability and no induction of cellular senescence. By γH2AX staining, DNA damage was observed in HERS cells treated with 0.5 µM cisplatin, but those cells did not reduce the viability of them. Cisplatin is an important anticancer drug that kills cancer cells by damaging their DNA; the formation of cisplatin-DNA adducts interferes with DNA replication and transcription. H2AX is a key factor in the process of repair for damaged DNA and is recruited to the damaged sites. In the presence of DNA damage, H2AX is phosphorylated on the 139th serine residue, termed γH2AX. Of the methods for detecting DNA damage of the cells, such as neutral elution, pulse field electrophoresis, and comet assays, γH2AX assay is a particularly precise method. Although nuclear γH2AX staining was found in all the cisplatin-treated cells, irrespective of cisplatin concentration, no reduction in cell viability was obtained only in the HERS cells treated with low concentration of cisplatin (0.5 µM and 1.0 µM). Our results that 0.5 µM cisplatin treatment triggers marked EMT in HERS cells suggest that EMT induction may depend on the degree of DNA damage caused by cisplatin treatment. Activation of a DNA damage response, including formation of DNA damage foci containing γH2AX staining at persistent DNA strand breaks is the major trigger for cell senescence. HERS cells treated with 0.5 µM cisplatin maintained a negative reaction with SA-β-Gal staining, a marker for cellular senescence, although the cells treated with other concentrations of cisplatin showed marked cytoplasmic staining with SA-β-Gal (data not shown). Therefore, the pre-senescence status of 0.5 µM cisplatin-treated cells could be susceptible for EMT induction in HERS cells.

In the present study, EMT induction was examined with a low concentration of cisplatin in addition to TGF-β treatment. TGF-β-induced EMT is a well-known and fundamental process in organogenesis and cancer progression. The HERS cells stimulated by cisplatin or TGF-β exhibited morphological and biochemical features of EMT; western blot assay showed that the expression of epithelial markers was decreased, while the expression of mesenchymal markers was increased, compared with than in controls. The exposure of the HERS cells to low concentration of cisplatin facilitated EMT. These findings of TGF-β-induced EMT in the HERS cells are in line with those reported by a previous study. As TGF-β-induced EMT appears to be related to the completion of root formation, chemotherapy-induced EMT may lead to the eruption disturbances via early closure of the apex in children undergoing chemotherapy.

Western blot assay and immunofluorescence staining revealed that HERS cells treated with 0.5 µM cisplatin showed enhanced activity of the Shh signaling pathway. The Shh pathway plays an important role during the processes of embryonic development and tumorigenesis. In the present study, we first detected an increase in the protein expression of Shh in HERS cells treated with 0.5 µM cisplatin. With immunofluorescence staining, we found that the nuclear translocation of Gli1 was significantly enhanced in the HERS cells exposed to cisplatin than in the controls. The nuclear localization and accumulation of Gli1 is essential for its transcriptional activity. Enhanced translocation of Gli1, a target of Shh, indicates the activation of the Shh signaling pathway. These findings suggest that the expression of Shh/Gli1 unregulated in response to cisplatin treatment. In our immunocytochemical staining, the number of Snail-expressed cells were significantly increased in the HERS cells treated with cisplatin. A recent study has emphasized the crucial roles of the transcription factor Snail during the EMT process. Snail is also the target of the Shh/Gli1 signaling pathway. Based on these findings, we suggest that cisplatin-induced EMT may be partially mediated by the Shh/Gli1 signaling pathway.

Gene silencing of Gli1 demonstrated that the activity of Shh/Gli1 signaling pathway can promote cisplatin-induced EMT in the HERS cells. Immunofluorescence assay showed that the percentages of E-cadherin-positive cells were decreased in cisplatin-treated cells in the presence of Gli1 siRNA than in the presence of negative control siRNA. In contrast, the percentages of vimentin-positive cells were increased in the Gli1 siRNA group. Furthermore, following the blockade of Gli1 expression by siRNA, cisplatin-treated HERS cells had lower cytoplasmic Snail expression. Based on these data, we proposed that the treatment with cisplatin activated Shh/Gli1 signaling pathway, which unregulated the nuclear translocation of Gli1 and in turn increased Snail expression, finally resulting in induction of EMT.

The present study has certain limitations, especially in terms of the lack of direct evidence regarding whether the blockade of Shh signaling can suppress the activity of the cisplatin-induced EMT. Immunocytochemical assay showed that silencing of Gli1, a target of Shh signaling inhibited the cisplatin-induced EMT. Further studies are required to examine the vital role of Shh in the cisplatin-induced EMT using pretreatment of cyclopamine, an Shh inhibitor. In this study, most data were obtained via immunofluorescence staining. We believe that protein expression data must be acquired using western blotting and used in the experiments of silencing or blockade of genes in further studies.

Thus, our findings provide additional information about the effect of anticancer drugs on EMT promotion in HERS cells. We demonstrated that low concentrations of cisplatin promote EMT in HERS cells via the activation of the Shh/Gli1 signaling pathway by inducing DNA damage.

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Conflict of Interest

The authors have no competing interest to declare.

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