Combination Cancer Therapy of a Del1 Fragment and Cisplatin Enhanced Therapeutic Efficiency *In Vivo*

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Abstract. *Background/Aim:* Combination cancer therapy is currently under investigation. This study examined the effect of cancer combination therapy using the E3 and C1 (E3C1) domains of developmental endothelial locus-1 (Del1) and cisplatin (CDDP) in murine transplanted tumors. *Materials and Methods:* Mice with transplanted tumors (A431, SCCN or SCC-4 cells) were injected intraperitoneally with CDDP and injected locally with nonviral plasmid vectors encoding E3C1. *Histochemical analysis of the transplanted tumors was then performed to assess the effects on prognosis. Results:* The CDDP+E3C1 injected group had reduced tumor growth and longer survival compared to the CDDP injected group. In addition, cell death was observed in the tumor of the CDDP+E3C1 group. Furthermore, angiogenesis and increased blood vessels were observed together with stromal development. *Conclusion:* The CDDP+E3C1 treatment resulted in improved survival and poor tumor stromal development in mice with transplanted tumors.

Cisplatin (CDDP) is currently the most essential anticancer agent in cancer chemotherapy. CDDP is used in most chemotherapy regimens for gastric, colorectal, lung and head and neck cancers. Furthermore, CDDP has shown efficacy against a wide variety of cancers. In addition, CDDP is also used as neo-adjunctive or adjunctive therapy for surgery. The history of CDDP as an anticancer agent began in 1965 when Rosenberg *et al.* (1) discovered the

inhibitory effects of the electrolytic products of platinum electrodes on cell division in *Escherichia coli* (2). CDDP is understood to exhibit anticancer activity by forming DNA and covalent adducts (3-5). In its mechanism of action, CDDP forms DNA and various covalent adducts, inhibiting transcription factors and polymerase and destroying chromatin. These activities eventually lead to cell apoptosis (6-9). Williams *et al.* reported that administering CDDP as a single agent had a 60% effective rate against testicular cancer, with 19% of cases showing complete tumor disappearance (10). Further, Panetti *et al.* reported a 25% effective rate for CDDP single-agent administration against head and neck cancer (11). In addition, combination chemotherapies using CDDP have been reported to show efficacy against squamous cell carcinoma (SCC) of 75% or more (12-15). On the other hand, CDDP is also known to be associated with various severe side effects (nephrotoxicity, nausea, vomiting, hearing loss, weight reduction, *etc.*) (16-18). In addition, CDDP resistance can be induced by CDDP treatment (19, 20). These events represent major disadvantages to CDDP treatment.

Currently, SCC is the most common cancer type in the world. The main treatments for SCC are surgery, chemotherapy, and radiotherapy. Cancer gene therapy is also under development as a new modality (21-25). In 1991, the first clinical trial of cancer gene therapy demonstrated that genetically modified immune cells could be reintroduced into patients, and with the approval of CAR-T cell therapy by the United States Food and Drug Administration (FDA), this field has seen tremendous advances (26, 27). Kitano *et al.* attempted gene therapy against SCC explants *in vivo* (28, 29). Mice with explanted SCC cells were treated with the extracellular matrix protein developmental endothelial locus-1 (Del1) using a non-viral vector. Del1 has been shown to exhibit pro- or anti-angiogenic activities (30). Del1 is comprised of five domains: three epidermal growth factor (EGF) repeats (E1, E2, and E3) and two discoidin domains (C1 and C2) (30). The E2 domain is reported to contain

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RGD sequences, which bind to integrin receptors and support endothelial cell survival (30). The E3 domain induces endocytosis and increases the efficiency of gene transfer, and has been reported to induce apoptosis (31, 32). Furthermore, the C1 domain has been reported to cause deposition in the extracellular matrix (33). A recombinant protein comprising the E3 and C1 domains (E3C1) of Del1 was thus hypothesized to be deposited in extracellular matrix at high concentrations, induce apoptosis, and enhance the efficiency of subsequent transfections. Mice with transplanted tumors therefore received weekly local injections using DNA encoding a recombinant protein of E3C1 by a non-viral vector. As a result, transplanted SCCN tumors showed efficient deposition of E3C1, and increased gene transfer efficiency (29). In addition, 50% of mice treated with E3C1 survived for 7 weeks, whereas all mice in the control group died prior to this time point (29). Furthermore, analysis of the effects of long-term E3C1 treatment showed that 20% of E3C1-treated mice survived for 197 days, with explanted tumors resolving by day 197 in two mice (34). With transplanted A431 tumors, 33% of mice treated with E3C1 survived more than 60 days, whereas all control mice died by day 32 (35).

Due to enhanced therapeutic response and minimal adverse effects, combination cancer therapy has recently become widespread all over the world. The aim of this study was to examine the effects of combination cancer therapy comprising cancer gene therapy using the EGF motif of Del1 and CDDP as the most commonly used chemotherapeutic agent for SCC cells. In this regard, mouse transplanted tumors were consecutively treated by E3C1 until a humane end point was reached. This study confirmed that combination therapy of CDDP+E3C1 inhibited the growth of SCC transplants and improved the survival of mice with transplanted tumors.

Materials and Methods

Cell lines and culture. The A431 human epidermoid squamous carcinoma cell line (ATCC: CRL-1555) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) (36, 37). This cell line was grown in serum-free medium [60% Opti-MEM (Invitrogen, Carlsbad, CA, USA), 40% LHC-8medium (Invitrogen)]. The SCCN (RIKEN BioResource Research Center, Tokyo, Japan, cell number: RCB0441) (38, 39) human tongue squamous carcinoma cell line was kindly provided by Dr. Hayashido from the University of Hiroshima (Hiroshima, Japan). This cell line was grown in RD medium, comprised of 45% Dulbecco's modified Eagle medium (Invitrogen), 45% RPMI 1640 medium (Invitrogen), and 10% fetal bovine serum (Sigma, St. Louis, MO, USA). Cells were then cultured in 5% CO₂ at 37°C. The SCC-4 human tongue squamous carcinoma cell line was purchased from ATCC (ATCC: CRL-1624) (40, 41). This cell line was grown in RPMI 1640 medium (Invitrogen) and 10% fetal bovine serum. Cells were cultured in 5% CO₂ at 37°C.

DNA constructs. Mouse Del1 complementary DNA was kindly provided by Dr. Quertermous of Stanford University (Stanford, CA, USA). A fragment encoding E3C1 (amino acids 122-316 of mouse Del1) was amplified using polymerase chain reaction with the forward primer 5'-TGTGAAGCTGAGCCTTGCAGAATGGCCGGA-3' and the reverse primer 5'-ACAGCCTGAGAGCTCACAGCCAAGAAGTT-3' to generate pcDNA3.1ID (Invitrogen), a mammalian expression vector. A signal peptide sequence of the E3C1-complementary DNA was then inserted into pcDNA3.1ID to generate a recombinant protein with V5-epitope tags at the C-terminal ends.

Animal studies. All animal experiments performed in this study were conducted in accordance with the animal husbandry regulations of Nihon University and the US National Institutes of Health. This study was approved by the Ethics Committee of Nihon University (AP18MED023-2). NOD CB17-Prkdc scid/J mice were kept in a pathogen-free environment. For tumor explants, A431, SCCN or SCC4 cells were first seeded in 10-cm culture dishes overnight to reach 70-80% confluence and were harvested using a cell stripper (Asone, Tokyo, Japan) after washing with phosphate-buffered saline (PBS). The cells were then washed twice with PBS and centrifuged at 500×g for 5 min, then resuspended in RD medium at a concentration of 1×10⁷/100 ml. A total of 100 µl of suspended cells were injected subcutaneously into the right flank of a NOD CB17-Prkdc scid/J mouse. Tumor growth was measured using calipers twice a week. Tumor volume was calculated using the following formula: tumor volume (in cubic millimeters)=(width×length²)/2. Mouse weights were calculated using the formula (whole weight – tumor volume). When tumor volume exceeded 60 mm³, treatment was initiated with CDDP (Fujifilm, Tokyo, Japan), CDDP together with the plasmid encoding E3C1 (CDDP+E3C1) or pcDNA3 as a mock vector for controls. As treatment, CDDP was injected intraperitoneally at 10 mg/kg and 10 µg of DNA was injected into the tumor in 100 µL increments using *in vivo*-jetPEI (Polyplus Transfection, San Marcos, CA, USA) once a week. When the tumor had grown to >15% of body weight, the mouse was considered to have reached a humane end point and was therefore euthanized. Eleven days after starting treatment, all remaining mice were sacrificed under deep anesthesia with isoflurane for histological examination. On euthanasia, the tumor was immediately removed and frozen at –80°C, after which, 5-mm frozen sections were prepared.

Evaluation of perfusion. Half of the mice transplanted tumor with SCC4 cells in each treatment group was injected intravenously with fluorescein-conjugated *Lycopersicon esculentum* (tomato) lectin (LEL) (0.4 mg/ml) (Vector Laboratories, Burlingame, CA, USA). The mice were euthanized 2 min after the injection. The transplanted tumors were immediately resected and placed in Optimal Cutting Temperature (OCT) compound (Sakura Finetek Japan, Tokyo, Japan). Blocks then were stored at –80°C. Furthermore, the other half of the mice received an intravenous injection of Black ink, and were then euthanized after 2 min. Transplanted tumors were immediately fixed in 4% paraformaldehyde (PFA).

Immunohistochemistry. Rabbit polyclonal anti-CD31 (PECAM) antibody or anti-alpha smooth muscle actin (αSMA) antibody were purchased from Abcam (Cambridge, UK). Alexa Fluor 568-labeled goat antirabbit antibodies were purchased from Invitrogen. For immunohistochemistry, 5-µm sections were fixed with 4% PFA, incubated with primary antibodies, and then incubated with the appropriate secondary antibodies. Hoechst 33342 was used to visualize nuclei. An Axioskop 2 microscope (Carl Zeiss

Figure 1. Effect of combination therapy for mice with transplanted A431 cells. (A) Kaplan-Meier survival curves of mice in the control group (solid line, n=7), CDDP treatment group (chain line, n=6) and CDDP+E3C1 treatment group (dashed line, n=7) (control vs. CDDP, $p<0.05$; control vs. CDDP+E3C1, $p<0.01$; CDDP vs. CDDP+E3C1, $p<0.05$). (B-D) Chronological changes in tumor volume in individual mice following control (B), CDDP treatment (C) and CDDP+E3C1 treatment (D). (E) After 11 days of treatment, comparison of tumor volumes (control vs. CDDP, $p<0.05$; control vs. CDDP+E3C1, $p<0.01$). (F) After 11 days of treatment, comparison of mouse weights. Results are expressed as mean $\pm$ SD (E, F).

Microimaging, Welwyn Garden City, UK) equipped with an AxioCam (Carl Zeiss Microimaging) was used to observe tissues and take photographs. Fluorescence intensity was measured using Photoshop v 7.0 (Adobe Systems Incorporated, San Jose, CA). The intensity in control samples was set as 1. Results per view are expressed as mean $\pm$ standard deviation (SD).

Statistical analysis. The survival analysis was performed by Kaplan-Meier log-rank test. One-way ANOVA and then Bonferroni correction were performed as appropriate. The results of except for

survival analyses are expressed as mean $\pm$ SD. The statistical significance was defined as * $p<0.05$, ** $p<0.01$. Data were analyzed using the SPSS software (version 16, Chicago, IL, USA).

Results

Combination therapy with CDDP+E3C1 prolonged survival in mice with A431-derived tumors. Mice with tumors from A431 cells were treated by control, CDDP, or CDDP+E3C1

Figure 2. Effect of combination therapy for mice with transplanted SCKN cells. (A) Kaplan-Meier survival curves of mice in the control group (solid line, $n=4$), CDDP treatment group (chain line, $n=4$) and CDDP+E3C1 treatment group (dashed line, $n=4$) (control vs. CDDP, $p<0.05$; control vs. CDDP+E3C1, $p<0.05$; CDDP vs. CDDP+E3C1, $p<0.05$). (B-D) Chronological changes in tumor volume in individual mice following control (B), CDDP treatment (C) and CDDP+E3C1 treatment (D). (E) After 11 days of treatment, comparison of tumor volumes (control vs. CDDP+E3C1, $p<0.05$; CDDP vs. CDDP+E3C1, $p<0.05$). (F) After 11 days of treatment, comparison of mouse weights (control vs. CDDP, $p<0.01$; control vs. CDDP+E3C1, $p<0.05$; CDDP vs. CDDP+E3C1, $p<0.01$). Results are expressed as mean $\pm$ SD (E, F).

once a week. All 20 mice (control group, $n=7$; CDDP group, $n=6$; CDDP+E3C1 group, $n=7$) were euthanized because the tumor grew to $>5,000$ mm³. Mice in the control group began to die from day 11, and all mice treated in this group died by day 25 (Figure 1A). Mice treated by CDDP began to die from day 18 and all mice treated in this group died by day 29 (Figure 1A). In contrast, mice treated by CDDP+E3C1

began to die from day 22 and all mice treated in this group died by day 36 (Figure 1A). Survival rates of mice treated by CDDP or CDDP+E3C1 were significantly higher than the survival rate of mice in the control group (control vs. CDDP, $p=0.049$; control vs. CDDP+E3C1, $p=0.009$). In addition, the survival rate was significantly higher for mice of treated by CDDP+E3C1 than for mice treated by CDDP ($p=0.048$).

Figure 3. Effect of combination therapy for mice with transplanted SCC4 cells. (A) Kaplan-Meier survival curves of mice in the control group (solid line, $n=6$), CDDP treatment group (chain line, $n=6$) and CDDP+E3C1 treatment group (dashed line, $n=5$) (Control vs. CDDP, $p<0.01$; Control vs. CDDP+E3C1, $p<0.01$; CDDP vs. CDDP+E3C1, $p<0.01$). (B-D) Chronological changes in tumor volume in individual mice following control (B), CDDP treatment (C) and CDDP+E3C1 treatment (D). (E) After 11 days of treatment, comparison of tumor volumes (control vs. CDDP+E3C1, $p<0.01$; CDDP vs. CDDP+E3C1, $p<0.01$). (F) After 11 days of treatment, comparison of mouse weights. Results are expressed as mean $\pm$ SD (E, F).

Transplanted tumors in mice treated by control consistently increased in volume over time (Figure 1B). The rate of increase in tumor volume was slower in mice treated by CDDP than in control mice. However, the tumor volume of mice treated by CDDP also increased over time (Figure 1C). The rate of increase in tumor volume was the slowest in mice treated by CDDP+E3C1, and 6 out of 7 tumors showed

temporary reductions in volume (Figure 1D). Mean tumor volume on day 11 of treatment was $3,149.071\pm1,139.171$ mm³ in control mice, $1,843.583\pm556.3021$ mm³ in mice treated by CDDP and 1514.071 ± 471.3341 mm³ in mice treated by CDDP+E3C1 (control vs. CDDP, $p=0.035$; control vs. CDDP+E3C1, $p=0.009$; CDDP vs. CDDP+E3C1, $p=0.284$) (Figure 1E). In addition, mean body weights of

Figure 4. Histopathological analysis of mouse tumors by combination therapy. After 11 days of treatment, transplanted tumors were stained with hematoxylin and eosin in the control group (A, B), CDDP treatment group (C, D) and CDDP+E3C1 treatment group (E, F). (A, C, E) Images were taken using a $\times 10$ objective lens. (A) Arrows indicate necrotic lesions. (B, D, F) Enlargements of regions outlined in (A), (C) and (E), respectively ($\times 20$). Scale bars, 50 μm . P: parenchyma; S: stroma; *cell death region.

mice on day 11 of treatment were 16.942 ± 1.919 g in control mice, 16.547 ± 2.773 g in mice treated by CDDP and 17.4 ± 2.238 g in mice treated by CDDP+E3C1 (control vs. CDDP, $p=0.394$; control vs. CDDP+E3C1, $p=0.384$; CDDP vs. CDDP+E3C1, $p=0.356$) (Figure 1F).

Combination therapy with CDDP+E3C1 prolonged survival in mice with SCCKN-derived tumors. Mice with tumors from SCCKN cells were treated by control, CDDP, or CDDP+E3C1 once a week. All 12 mice (control group, $n=4$; CDDP group, $n=4$; CDDP+E3C1 group, $n=4$) were euthanized because tumors grew to $>5,000$ mm³. Mice in the control group began to die on day 11 and all mice died by day 18 (Figure 2A). On the other hand, mice treated by CDDP began to die on day 22 and all mice died by day 25 (Figure 2A). Mice treated by CDDP+E3C1 began to die on day 32 and all mice died by day

39 (Figure 2A). The survival rates of mice treated by CDDP or CDDP+E3C1 were significantly higher compared to control mice (control vs. CDDP, $p=0.035$; control vs. CDDP+E3C1, $p=0.023$). In addition, the survival rate of mice treated by CDDP+E3C1 was significantly higher than that of mice treated by CDDP alone ($p=0.031$). Transplanted tumors in control mice consistently increased in volume over time (Figure 2B). The rate of increase in tumor volume for mice treated by CDDP was slower compared to control mice. However, the tumor volume in mice treated by CDDP increased over time (Figure 2C). Two of the four tumors temporarily showed a reduction in volume (Figure 2C). The rate of increase in tumor volume in mice treated by CDDP+E3C1 was the slowest of the three groups. Three of the four tumors temporarily decreased in volume (Figure 2D). Mean tumor volume on day 11 of treatment was $3,990.625 \pm 1,403.377$ mm³ in mice treated with

control, $3,468.375 \pm 757.457 \text{ mm}^3$ in mice treated by CDDP and $1,177.875 \pm 481.35 \text{ mm}^3$ in mice treated by CDDP+E3C1 (control vs. CDDP, $p=0.539$; control vs. CDDP+E3C1, $p=0.045$; CDDP vs. CDDP+E3C1, $p=0.041$) (Figure 2E). In addition, the mean weight of mice on day 11 of treatment was $17.809 \pm 0.948 \text{ g}$ in mice treated by control, $13.282 \pm 0.589 \text{ g}$ in mice treated by CDDP and $15.697 \pm 1.365 \text{ g}$ in mice treated by CDDP+E3C1 (control vs. CDDP, $p=0.0003$; control vs. CDDP+E3C1, $p=0.042$; CDDP vs. CDDP+E3C1, $p=0.009$) (Figure 2F).

Combination therapy with CDDP+E3C1 prolonged survival in mice with SCC4-derived tumors. Mice with tumors from SCC4 cells were treated by control, CDDP, or CDDP+E3C1 once a week. All 17 mice (control group, $n=6$; CDDP group, $n=6$; CDDP+E3C1 group, $n=5$) were euthanized because the tumor grew to $>5,000 \text{ mm}^3$. Mice in the control group began to die from day 15 and all mice died by day 18 (Figure 3A). Mice treated by CDDP began to die from day 22 and all mice died by day 25 (Figure 3A). Mice treated by CDDP+E3C1 began to die from day 25 and all died by day 36 (Figure 3A). The survival rates of mice treated by CDDP or CDDP+E3C1 were significantly higher compared to controls (control vs. CDDP, $p=0.009$; control vs. CDDP+E3C1, $p=0.008$). In addition, the survival rate of mice treated by CDDP+E3C1 was significantly higher than that of mice treated by CDDP ($p=0.009$). Transplanted tumors in mice treated by control showed consistent increases in tumor volume over time (Figure 3B). The rate of increase in tumor volume was slower for mice treated by CDDP compared to controls (Figure 3C). However, two of the six tumors showed temporary reductions in volume (Figure 3C). The rate of increase in tumor volume in mice treated by CDDP+E3C1 was the slowest of the three groups. Three out of five tumors showed temporary reductions in volume (Figure 3D). Mean tumor volume on day 11 of treatment was $2,702 \pm 394.405 \text{ mm}^3$ in control mice treated, $3,135.5 \pm 870.581 \text{ mm}^3$ in mice treated by CDDP and $619.1 \pm 105.532 \text{ mm}^3$ in mice treated by CDDP+E3C1 (control vs. CDDP, $p=0.982$; control vs. CDDP+E3C1, $p=0.009$; CDDP vs. CDDP+E3C1, $p=0.008$) (Figure 3E). In addition, the mean weight of mice on day 11 of treatment was $18.115 \pm 1.944 \text{ g}$ in control mice, $17.048 \pm 2.78 \text{ g}$ in mice treated by CDDP and $18.721 \pm 1.484 \text{ g}$ in mice treated by CDDP+E3C1 (control vs. CDDP, $p=0.453$; control vs. CDDP+E3C1, $p=0.39$; CDDP vs. CDDP+E3C1, $p=0.255$) (Figure 3F).

Combination therapy showed histopathological effects in transplanted tumors. Sections from transplanted tumors of SCC4 on day 11 of treatment were stained with hematoxylin and eosin for observation (Figure 4A-F). Tumors from control mice showed parenchyma and stroma, with little cell death (Figure 4A). Some sites of cell death were apparent

(Figure 4A arrow). Tumor stroma had developed (Figure 4B). By contrast, tumors treated by CDDP showed cell death in many parts of the tumor parenchyma and stroma (Figure 4C). Parenchyma was observed around the stroma (Figure 4D). Furthermore, parenchyma and stroma were neatly arranged in tumors treated by CDDP+E3C1 (Figure 4E). In addition, compared to tumors in the control or CDDP groups, the stroma was arranged regularly in a characteristic direction (Figure 4F).

Combination therapy resulted in improvement in the stroma of transplanted tumors. Next, mice with SCC4-derived tumors received intravenous injection of LEL via the caudal vein on day 11. Sections from the tumors were stained with anti-aSMA. In control mice, anti-aSMA-positive cells were identified throughout the entire tumor stroma (Figure 5B). In addition, aSMA expression (Figure 5C) was found in the periphery of vessels filled by LEL (Figure 5A). In contrast, tumors in mice treated by CDDP showed lower expression of aSMA in the tumor stroma (Figure 5E), and little aSMA expression (Figure 5F) around vessels filled by LEL (Figure 5D). Tumors in mice treated by CDDP+E3C1 showed suppression of aSMA expression (Figure 5H). Furthermore, aSMA expression (Figure 5I) was also suppressed around vessels filled by LEL (Figure 5G). The fluorescent intensity of aSMA was 1 ± 0.12 in the control group, compared to 0.32 ± 0.01 in the CDDP group and 0.25 ± 0.03 in the CDDP+E3C1 group (control vs. CDDP, $p=0.00006$; control vs. CDDP+E3C1, $p=0.00006$; CDDP vs. CDDP+E3C1, $p=0.007$) (Figure 5J).

Combination therapy caused transformation in tumor angiogenesis in the transplanted tumors. Mice with SCC4-derived tumors received intravenous injections of Indian ink via the caudal vein on day 11. In control mice, tumor vessels with numerous developing branches were observed (Figure 6A). In addition, a number of thin blood vessels were observed (Figure 6A). Tumor vessels were also reduced in number and branches, with no thick blood vessels observed in mice treated by CDDP (Figure 6B). On the other hand, thick blood vessels were seen following treatment by CDDP+E3C1 (Figure 6C). However, the number of thin vessels and branches was reduced compared to tumor vessels in mice treated by control (Figure 6C).

In addition, tumor sections were stained using PECAM as an endothelial cell marker. In tumors from mice treated by control, expression of PECAM was observed in the tumor stroma (Figure 7A-C). Expression of PECAM in the stroma was also observed after treatment with CDDP, but the expression rate was lower than that in control mice (Figure 7D-F). On the other hand, tumors in mice treated by CDDP+E3C1 showed expression of PECAM in the tumor stroma similar to that of controls (Figure 7G-I). In addition, vascularization was evident in the stroma [Figure 7G (arrow)].

Figure 5. Immunohistochemistry analysis of mouse transplanted tumor by combination therapy. After 11 days of treatment, mice with transplanted tumors were injected with *Lycopersicon esculentum* lectin (LEL) after each treatment (green). Specimens of transplanted tumors from mice were stained by immunohistochemistry using anti-aSMA antibody (Red). Control (A-C), CDDP treatment group (D-F), CDDP+E3C1 treatment group (G-I). Magnification is $\times 20$. Scale bars correspond to 50 μm . The ratio for the control was taken as 1 (control vs. CDDP, $p < 0.01$; control vs. CDDP+E3C1, $p < 0.01$; CDDP vs. CDDP+E3C1, $p < 0.01$). Results are expressed as mean $\pm$ SD (J).

Figure 6. Tumor angiogenesis analysis of mouse transplanted tumor by combination therapy on angiography. After 11 days of treatment, mice with transplanted tumors were injected with India ink after each treatment. Control group (A), CDDP treatment group (B) and CDDP+E3C1 treatment group (C). Scale bars, 50 μ m.

Discussion

Currently, chemotherapy with CDDP, bleomycin, and methotrexate is effective in the treatment of SCC, and polypharmacy, including CDDP, has been reported to have a high response (42, 43). Kitano *et al.* reported that cancer gene therapy with the E3C1 domain of Del1 was effective in the treatment of SCC (28, 29). In this study, chemotherapy with CDDP and cancer gene therapy with E3C1 for mice that had received transplants of SCC was conducted to determine the therapeutic effects. The carcinoma cell lines used for transplantation into mice were human epidermoid SCC cells (A431), human oral SCC cells (SCCKN) and human oral SCC cells (SCC4). Treatment of A431, SCCKN, and SCC4-derived tumors with CDDP reduced tumor growth rates compared to controls. Survival was extended by 4 days in the case of A431, 7 days in SCCKN and 7 days in SCC4, compared to controls. In addition, treatment with CDDP+E3C1 further reduced the rate of tumor growth compared to the treatment with CDDP. Survival was extended by 7 days for A431, 14 days for SCCKN, and 9 days for SCC4, compared to treatment with CDDP alone. These results show that the combination of CDDP+E3C1 is much more effective in suppressing tumor growth and improving the survival of mice with transplanted SCC cells, compared to CDDP treatment alone. In addition, on day 11 of treatment, comparison of mouse body weights showed that mice treated with CDDP had lost more weight than the control, but those treated by CDDP +E3C1 had gained weight compared to controls. This result suggested that E3C1 suppressed the problematic side effects (weight reduction) of treatment by CDDP. The addition of E3C1 morphologically normalized the development of stroma and vessels. Suppression of weight reduction could be through E3C1 that might improve metabolism in stroma against the adverse effects of CDDP. However, the mechanisms involved remain unclear.

Next, analyses were conducted to clarify the reasons behind the greater effectiveness of combined CDDP and

E3C1 treatment, compared to CDDP treatment alone, in suppressing tumor growth. In observation of the tumor on day 11 of treatment, the treatment by CDDP showed cell death in many parts of the tumor parenchyma and stroma, while stroma in the remaining parts of the tumor was underdeveloped. On the other hand, stroma in the remaining part of the tumor was more developed in the CDDP +E3C1 group compared to controls. The primary purpose of cancer chemotherapy is to kill cancer cells by acting directly on the tumor parenchyma. Histological evaluation of the effect of chemotherapy has thus been focusing on changes in cancer parenchyma, such as degeneration and necrosis of cancer cells (44). However, more recently, the stroma of cancer tumors has received increased attention, as the extracellular matrix has been actively studied and its biological significance debated (45, 46). Tissue specimens have been observed in detail, showing that treatment by CDDP causes degeneration and necrosis not only in the cancer parenchyma, but also in the interstitial space around the oncocyst. However, stromal development was observed in mice treated by CDDP E3C1, suggesting that E3C1 repaired or altered the CDDP-denatured stroma.

Stromal cells in tumors are closely related to cancer growth and metastasis by producing cell-to-cell contacts and various extracellular matrix proteins (47-49). Stromal cells include fibroblasts, vascular endothelial cells, antigen-presenting cells, innate immune cells, and acquired immune cells. In cancer, the stroma reportedly maintains an active phenotype and establishes a favorable microenvironment for cancer progression through promotion of cancer cell proliferation, angiogenesis, vascular invasion by cancer cells and control of the immune response (50). In addition, stromal factors reportedly correlate with prognosis in different cancers including head and neck, lung, colon cancer and others (51-53). Denatured stroma from the combination treatment with CDDP+E3C1 was thus considered to have affected the growth and angiogenesis of cancer cells.

Figure 7. Immunohistochemical analysis of tumor angiogenesis of mouse tumors with combination therapy. After 11 days of treatment, the tumor specimen was stained using an anti-PECAM antibody (red) and Hoechst33342 (blue). Control group (A-C), CDDP treatment group (D-F), CDDP+E3C1 treatment group (G-I). Arrows indicate vascular lumen (G). Magnification is $\times 20$. Scale bars correspond to 50 μm .

Typically, angiogenesis occurs in the peritumoral area as the tumor increases in size. Necrosis of tumor cells due to blood flow arrest is repeated inside the tumor (54, 55). Fujisawa (56) reported how CDDP affects angiogenesis in cancer, but details of the association between CDDP and angiogenesis have not been clarified. In our study, the cancer stroma degenerated with treatment using CDDP. Vascular endothelial cells were considered to have been damaged by CDDP treatment and angiogenesis was reduced. Tumor vessels present in the stroma of each treatment group were thus observed. Fewer tumor vessels were seen in the group treated by CDDP compared to control. In addition, tumor vessels in the CDDP group were less well developed than in controls. This suggests that CDDP treatment caused degeneration and necrosis of the tumor stroma, resulting in the destruction of blood vessels. On the

other hand, CDDP+E3C1 tumor vessels were thicker and more developed, with numerous vascular branches. In addition, stromal degeneration attributed to CDDP treatment was restored and redeveloped by the effect of E3C1. Angiogenesis was thus thought to be accelerated. In short, vessel transfer and distribution with stromal development were observed in each treatment group. However, the results appear inexplicable and contradictory given the reduced tumor growth rate and prolonged life expectancy of mice treated by CDDP +E3C1.

The relationship between tumor vessels and tumor stroma in each treatment group was therefore examined by immunostaining for αSMA , an actin stress fiber formed in cells during fibroblast activation. This protein plays an important role in the formation of cell motility and is widely used as a marker of cancer stroma (57, 58). In head and neck,

lung, colon and various other cancers, α SMA expression has been reported to correlate with prognosis (53, 59-61). Expression of α SMA was somewhat suppressed by CDDP treatment, but CDDP+E3C1 treatment completely suppressed α SMA expression. In addition, tumors were immunostained with PECAM, a marker for vascular endothelial cells, and angiogenesis was examined. Angiogenesis and proliferation were both seen to be inhibited in by CDDP treatment, whereas treatment by CDDP+E3C1 showed significant angiogenesis and proliferation. This result was reviewed in conjunction with the results for α SMA and H-E staining. The stroma that developed under CDDP+E3C1 treatment was considered to represent normal tissue stroma instead of cancer stroma. The angiogenesis seen with CDDP+E3C1 treatment would thus reflect that in normal tissues.

These results were considered to indicate that E3C1 restores or alters the CDDP-degenerated interstitium, but the altered interstitium represents normal tissue interstitium and is thus recruited to tumor control. Currently, the stroma of carcinoma is considered to play a very important role in the process of SCC invasion into surrounding tissues (62, 63). In normal tissues, the stroma remains homeostatic. However, cancer stroma promotes invasion of cancer cells and angiogenesis (64, 65). In this study, the cancer stroma altered by CDDP+E3C1 treatment was well-developed and there was active angiogenesis and proliferation. However, the stroma altered by E3C1 was not repaired as cancerous interstitium, but instead was transformed into normal tissue stroma with the function of maintaining homeostasis. These findings suggest that the tumor growth rate was controlled and mice transplanted with tumors achieved an extended survival. The stroma of cancers treated with E3C1 should thus be investigated in more detail in the future.

Conclusion

In SCC, CDDP+E3C1 treatment was more effective than CDDP treatment alone for inhibiting tumor growth and prolonging survival. CDDP+E3C1 treatment was considered to transform the cancer stroma back into normal stroma. In addition, E3C1+CDDP treatment prevented the side effects (weight reduction) of CDDP treatment.

Conflicts of Interest

There Authors have no conflicts of interest to declare.

Authors' Contributions

Hisataka Kitano, You Masaoka, and Yusuke Fujiwara performed the research; Hisataka Kitano and Chiaki Hidai designed the research study; Hisataka Kitano, You Masaoka, Atsushi Mamiya, and Toshio Miki contributed essential reagents or tools; Hisataka Kitano and Chiaki Hidai wrote the paper.

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