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Release of Cytokines from Human Umbilical Vein Endothelial Cells Treated with Platinum Compounds *in vitro*

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Endothelial cells (EC) produce cytokines, such as interleukin (IL)-1, IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF). These cytokines have an important role in the proliferation and differentiation of hematopoietic progenitor cells. On the other hand, anticancer agents generally cause hematopoietic disorders. However, little is known about the effects of chemotherapeutic agents on the secretion of cytokines from EC. Therefore, we investigated if treatment with platinum compounds may stimulate EC to secrete cytokines. EC newly isolated from a human umbilical vein were exposed to cisplatin, carboplatin, or TRK-710 for 80 min, then the cells were washed and placed in fresh medium. The levels of cytokines in the fresh medium were measured by the ELISA method, the levels of intracellular hydrogen peroxide (H_2O_2) were measured by flow cytometry, and the rhodamine 123-stained live mitochondria of the EC were observed under a confocal laser microscope. Platinum compounds induced cytokine production in human EC: cisplatin most prominently induced the release of IL-1 and IL-6, and TRK-710 had the greatest ability to induce the release of GM-CSF. Intracellular H_2O_2 production and IL-8 release were transiently induced immediately after treatment with platinum compounds, leading to IL-1 release when H_2O_2 production was eliminated. These results may provide new insights into the hematological toxicity induced by anticancer agents and the role of IL-1 and IL-6 secreted from EC in this toxicity.

Key words: Cytokine — Hydrogen peroxide — Endothelial cell — Platinum compound

Endothelial cells (EC) are the primary physical barrier between blood and tissue in microvessels[1)] and have an important role in inflammation, shock and ischemia through endothelial-leukocyte interaction mediated by cytokines, chemotactic factors, other chemical mediators, and adhesion molecules.[2, 3)] On the other hand, microvascular EC and hemopoietic cells are embryologically considered to be differentiated from hemangioblasts.[4)] In fact, EC contribute to hematopoiesis by releasing cytokines that affect the proliferation and differentiation of hematopoietic progenitor cells.[5, 6)]

EC secrete cytokines, such as interleukin (IL)-1, IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF). IL-1, a key mediator in the cytokine network, alters many functions of blood vessel wall cells and induces production of endothelin by EC.[7)] It also influences *in vitro* growth of hemopoietic stem cells synergistically with hemopoietic growth factors.[8)] When stimulated by IL-1, EC produce high levels of IL-6[9, 10)]; anti-IL-6 antibodies do not affect the activity of IL-1.[10)] IL-6 also acts synergistically to support the proliferation of hemopoietic progenitors, and has an important role in producing hemopoietic cells, possibly at the level of the primitive pluripotent stem cell.[11)] The EC secrete IL-8, a neutrophil-activating cytokine,[12–14)] that acts as a mediator of inflammation,[15)] reduces chemotactic activity,[16)] and elicits leukocyte extravasation.[14, 17)] The hematopoietic cytokine, GM-CSF, increases erythroid and multipotential colony formation in enriched human bone marrow and peripheral blood progenitor cell cultures containing erythropoietin,[18–21)] and is both an effective stimulator of leukocytosis[22)] and an activator of the phagocytic function of mature neutrophils *in vivo*.[23, 24)]

The platinum coordination complexes represent the most important group of agents now in use for cancer treatment. They are curative in combination therapy for testicular cancer and ovarian cancer and have a central role in treating lung, head and neck, and bladder cancers.[25)] Cisplatin was the first platinum coordination drug used to treat testicular and ovarian tumors.[26)] Carboplatin, which was developed as a second-generation analog of cisplatin, has demonstrated significantly less nephrotoxicity and emetogenic potential in both preclinical and clinical studies.[27–29)] Moreover, a phase I trial in Japan showed that the new platinum analogue, TRK-710, has a lesser degree of nephrotoxicity and myelosuppression.[30)] Both cisplatin and carboplatin cause hematopoietic disorders as side effects,[26–29)] and they are administered intravenously.

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The degree of hematotoxicity induced by a particular agent is determined by multiple factors: compliance in taking the drug, variability of drug absorption, active gastrointestinal disease, previous surgery or radiation, concurrent medications that inhibit absorption, altered protein binding in plasma, compartment fluid excess, alterations in drug metabolism, and factors affecting drug excretion.[31] Furthermore, we supposed that cytokines secreted from venous EC as a consequence of the drug stimulation might have an important role in bone marrow injury and recovery. However, little is known about the role of EC in the hematopoietic disorders.

Therefore, we investigated if treatment with the platinum compounds may stimulate EC to secrete cytokines. In this paper, we describe the results of this investigation and discuss the role of EC in hematopoietic disorders induced by platinum compounds.

MATERIALS AND METHODS

Isolation of human endothelial cells from the umbilical vein We used human umbilical veins, because umbilical vein EC have the capability to grow further to increase in numbers. The EC were isolated from the umbilical vein within 2 h of delivery. The vein was cannulated at one end with a handmade blunt needle (1.1 mm outer diameter) and was secured by clamping the cord over the needle with an umbilical cord clamp (Atom Medical, Tokyo). The vein was perfused with Dulbecco's phosphate-buffered saline (PBS) until the solution was free of red blood cells, and then the vein was filled with 25 ml of trypsin solution [250 mg trypsin (17072-026, Gibco, Grand Island, NY) and 20 mg disodium dihydrogen ethylenediaminetetraacetate in 100 ml of PBS]. The cord was incubated at 37°C for 30 min, then the effluent was collected in a 50 ml conical centrifuge tube by flushing the vein once with 20 ml of MCDB 131 medium (10372-019, Life Technologies, Grand Island, NY)[32] containing 43.8 mg L-glutamine (710S1760, Kanto Chemical, Tokyo) and 10% fetal bovine serum. The cells were washed, and 10 ml of fresh MCDB medium was added. The cells (1×10^5/ml) were cultured in a 24-well tissue culture dish (Becton Dickinson, Lincoln Park, NJ) at 37°C under a 5% CO_2 atmosphere.

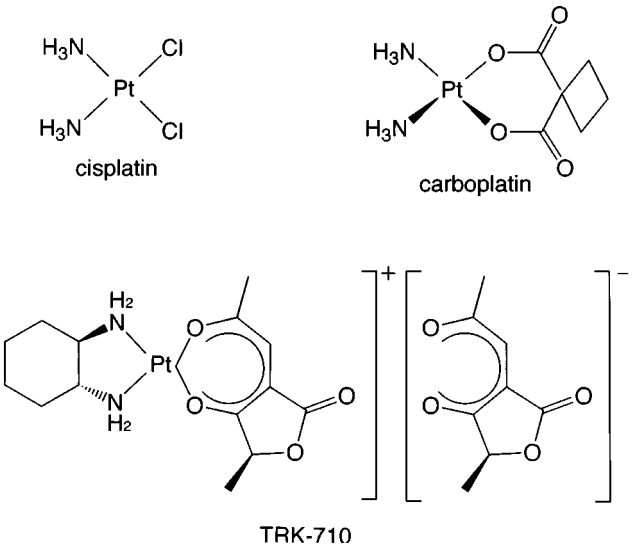

Fig. 1. Chemical structures of cisplatin, carboplatin and TRK-710. Carboplatin has a cyclobutanedicarboxylato residue attached to *cis*-diamine platinum. TRK-710 has a [(5S)-3-acetyl-5-methyl-2,4(3H,5H)-furandionate-O^3,O^4] ligand structure combined with *cis*-diamine platinum. This ligand may act in the cytoplasm after its dissociation.

Drug exposure The cells were preincubated for 48 h to allow them to adhere to the bottom of the wells, and then the culture medium was replaced with 0.45 ml of fresh MCDB medium, followed by addition of 50 μl of PBS, cisplatin (Bristol-Myers Squibb, Tokyo), carboplatin (Bristol-Myers Squibb), or TRK-710 (Toray, Tokyo) (Fig. 1). As the number of EC was insufficient to evaluate a dose-effect relationship, and as the purpose of this study was to see if EC treated with the three platinum compounds secrete cytokines, we chose the area under the curve (AUC) of serum concentration versus time as a parameter, because the AUC represents the total drug exposure integrated over time and is not affected by changes in drug schedule.[33] The concentration of each drug was calculated from the AUC data in the phase I trial: drug concentrations at AUC/80 min were 2.30 μg/ml for cisplatin,[34] 86.76 μg/ml for carboplatin,[35] and 1.68 μg/ml for TRK-710 (unpublished data). After 80 min incubation, the cells were washed once with PBS, 0.8 ml of MCDB medium was added to each well, and then incubation was continued for a further 1, 2, 4, 8, 24 or 48 h.

Measurement of human IL-1β, IL-6, IL-8 and GM-CSF by ELISA The IL-1β, IL-6, IL-8 and GM-CSF levels were measured using a CytoscreenTM immunoassay kit (BioSource International, Camarillo, CA). The specimens and control medium (50 μl to measure IL-1β and IL-8 and 100 μl to measure IL-6 and GM-CSF) were added to the appropriate microtiter antibody-coated wells. The 92-well culture plate was incubated at room temperature for 1.5 h to measure GM-CSF and at 37°C for 3 h to measure IL-6. The plate was washed four times with a buffer, then biotin-conjugate secondary antibodies were added to each well, and the plate was incubated at room temperature for 1 h, 45 min, 1.5 h or 1 h to measure IL-1β, IL-6, IL-8 and GM-CSF, respectively. It was again washed, then streptavidin-HRP working conjugate solution was added to each well, and the plate was further

incubated at room temperature for 30 min to measure IL-1β and IL-8 or for 45 min to measure IL-6 and GM-CSF. It was again washed, then stabilized chromogen was added to each well, and the plate was further incubated in the dark at room temperature for 20 min, 25 min, 25 min or 8 min for IL-1β, IL-6, IL-8 or GM-CSF, respectively. After addition of stop solution, the absorbance was measured using a Softmax Vmax kinetic microplate reader at the wavelength of 450 nm. The level of each cytokine secreted into the medium by EC was calculated by use the following equation: level of each cytokine secreted by EC into the medium = (level of each cytokine secreted by EC treated with platinum compound)−(level of each cytokine secreted by the untreated control EC).

Hydrogen peroxide (H_2O_2) Because PC-3 adenocarcinoma cells showed typical H_2O_2 production after the exposure of the cells to platinum compounds in our preliminary study, we used PC-3 cells as positive control cells. The H_2O_2 production in the cells exposed to each platinum compound was measured using a cytometer (Epics Elite, Coulter, Miami, FL) with a 488 nm excitation argon ion laser. Dichlorofluorescein diacetate (DCFH-DA, Eastman Kodak, Rochester, NY) incorporated into cells is metabolized to 2′,7′-dichlorofluorescein (DCFH), a nonpolar and a non-fluorescent compound, and H_2O_2 converts the DCFH to a polar and highly fluorescent compound, dichlorofluorescein (DCF).[36] Fluorescence of DCF was collected through a 530/30 nm band pass filter. Because the amount of intracellular DCF fluorescence indicates the level of H_2O_2, intracellular H_2O_2 levels were expressed as the mean cellular fluorescence (MCF) value in arbitrary units (AU) of a logarithmic scale. Fluorescent beads (CaliBRITE Beads, Becton Dickinson) were used to standardize and control the flow cytometer by optimizing the alignment, reproducing specific operating conditions, calibrating intensity scales, comparing sensitivities, and monitoring the instrument performance. The level of intracellular H_2O_2 after treatment of EC or PC-3 cells with a platinum compound was calculated by use of the equation: level of intracellular H_2O_2 after treatment of EC or PC-3 cells with platinum compound=(level of intracellular H_2O_2 produced in EC or PC-3 cells treated with platinum compound)/(level of intracellular H_2O_2 produced in untreated control EC or PC-3 cells).

Mitochondria Because mitochondria produce H_2O_2, we observed the morphological effects of platinum compounds on mitochondria. Live mitochondria were stained with rhodamin 123 (Rho) as described by Inoue *et al*.[37] Briefly, 24 h after drug exposure, Rho was added to the medium (final concentration of 10 μg/ml) and the cells in the monolayer were exposed to Rho for 30 min at 37°C. The cells were then washed with PBS, kept in PBS on ice, and observed as soon as possible using a confocal laser-scanning microscope (Bio-Rad Microscience Division, Watford, UK) equipped with a computer (NEC, Tokyo).

RESULTS

Confirmation of endothelial cells Because EC contain von Willebrand factor in the cytoplasm, the cells were stained with anti-human von Willebrand factor antibody by the ABC method[38] (data not shown). After confirming the presence of von Willebrand factor, we used the isolated cells as endothelial cells. Typical EC were homogeneously flat and thin cells with indistinct cell borders

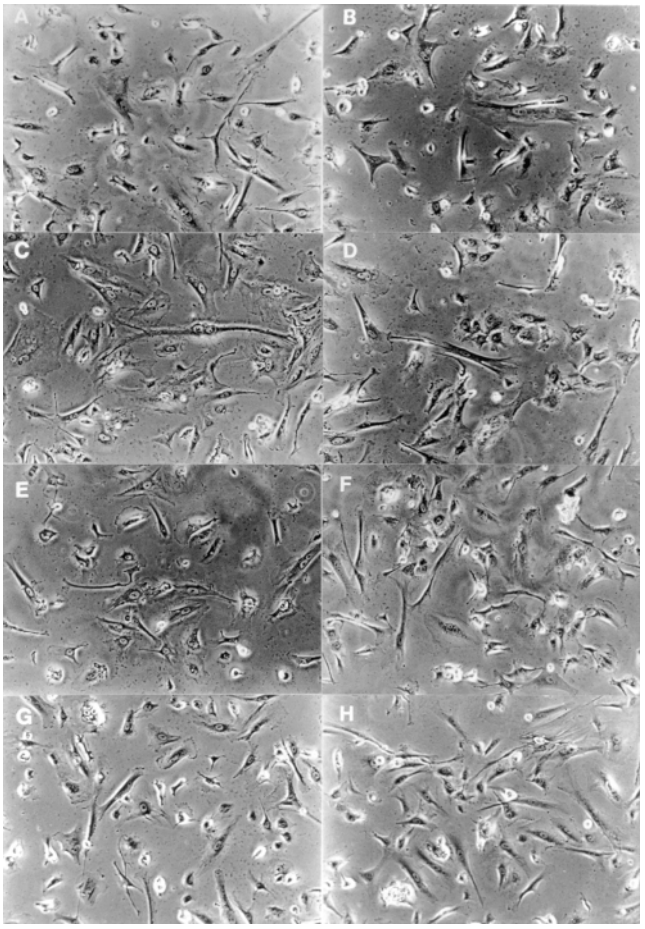

Fig. 2. Morphological changes after the exposure of EC to platinum compounds. The EC were exposed to each platinum compound for 80 min, washed free of the drug, and then reincubated in fresh medium supplemented with 10% fetal bovine serum. After incubation for 1, 2, 4, 8, 24 and 48 h, samples were observed under a phase-contrast microscope. The EC treated with cisplatin (C and D), carboplatin (E and F) or TRK-710 (G and H) did not differ morphologically from untreated control EC (A and B) at 24 h (A, C, E and G) or at 48 h (B, D, F and H).

Table I. Levels of IL-1, IL-6, IL-8 and GM-CSF Secreted into Medium[a)] and Intracellular H_2O_2 Levels in EC after Treatment with Cisplatin

Exposure time (h)	IL-8 (ng/ml)	H_2O_2 (AU)	IL-1 (ng/ml)	IL-6 (ng/ml)	GM-CSF (ng/ml)
1	−11.13±0.38	1.32±0.20	−12.73±2.86	−2.04±0.11	6.30±1.28
2	53.87±1.85	0.88±0.22	−14.83±0.88	−1.82±0.35	10.03±0.82
4	28.91±1.22	1.72±0.92	−8.13±2.84	−1.09±0.31	0.05±0.01
8	23.19±1.05	1.33±0.43	−3.83±0.20	1.17±0.22	−2.26±0.28
24	13.34±1.12	1.09±0.45	18.76±2.54	11.70±0.34	1.45±0.30
48	12.93±0.48	1.77±0.71	53.61±5.68	33.52±1.41	7.94±1.45

Values=mean±standard deviation.
a) Expressed as (level secreted by EC treated with cisplatin) − (level secreted by untreated control EC).

Table II. Levels of IL-1, IL-6, IL-8 and GM-CSF Secreted into Medium[a)] and Intracellular H_2O_2 Levels in EC after Treatment with Carboplatin

Exposure time (h)	IL-8 (ng/ml)	H_2O_2 (AU)	IL-1 (ng/ml)	IL-6 (ng/ml)	GM-CSF (ng/ml)
1	−12.62±1.17	0.94±0.41	−11.21±2.14	−2.38±0.30	14.65±0.70
2	36.15±2.04	1.17±0.18	−12.40±2.96	−1.84±0.19	15.92±0.49
4	18.67±1.98	1.43±0.66	−13.42±1.90	−1.15±0.09	14.61±0.58
8	12.76±0.57	1.28±0.48	−5.67±3.58	0.86±0.08	6.41±1.12
24	10.95±0.95	0.95±0.48	4.74±1.67	7.7±0.48	12.65±0.80
48	9.05±0.98	1.82±0.89	32.61±3.36	19.57±1.10	21.32±0.29

Values=mean±standard deviation.
a) Expressed as (level secreted by EC treated with carboplatin) − (level secreted by untreated control EC).

Table III. Levels of IL-1, IL-6, IL-8 and GM-CSF Secreted into Medium[a)] and Intracellular H_2O_2 Levels in EC after Treatment with TRK-710

Exposure time (h)	IL-8 (ng/ml)	H_2O_2 (AU)	IL-1 (ng/ml)	IL-6 (ng/ml)	GM-CSF (ng/ml)
1	−20.95±1.07	0.96±0.44	−12.95±2.13	−2.87±0.42	31.07±1.43
2	23.23±1.09	1.53±0.43	−14.32±2.57	−1.89±0.05	40.60±1.19
4	12.35±1.04	1.52±0.89	−15.79±1.03	−1.36±0.09	18.95±0.23
8	8.68±0.51	1.26±0.66	−11.46±2.26	0.01±0.00	26.72±0.96
24	7.23±0.80	2.64±1.16	−2.65±1.01	5.34±0.43	38.68±1.71
48	6.38±1.38	2.10±0.60	26.75±1.87	11.34±0.50	62.24±0.96

Values=mean±standard deviation.
a) Expressed as (level secreted by EC treated with TRK-710) − (level secreted by untreated control EC).

under a phase-contrast microscope (Fig. 2, A and B). The total cell number of EC three weeks after the isolation was 2.77×10^5/ml, and they were all used in the experiment.

Morphological changes after treatment with platinum compounds Although we initially attempted to evaluate the effect of three platinum compounds on cell growth by a colorimetric method, such as MTT or XTT assay, EC lacked the ability to convert MTT and XTT to colored formazan. Similarly, as EC did not grow rapidly, we could not measure the IC_{50}s. Therefore, the morphology of the EC was observed at 1, 2, 4, 8, 24 and 48 h after treatment

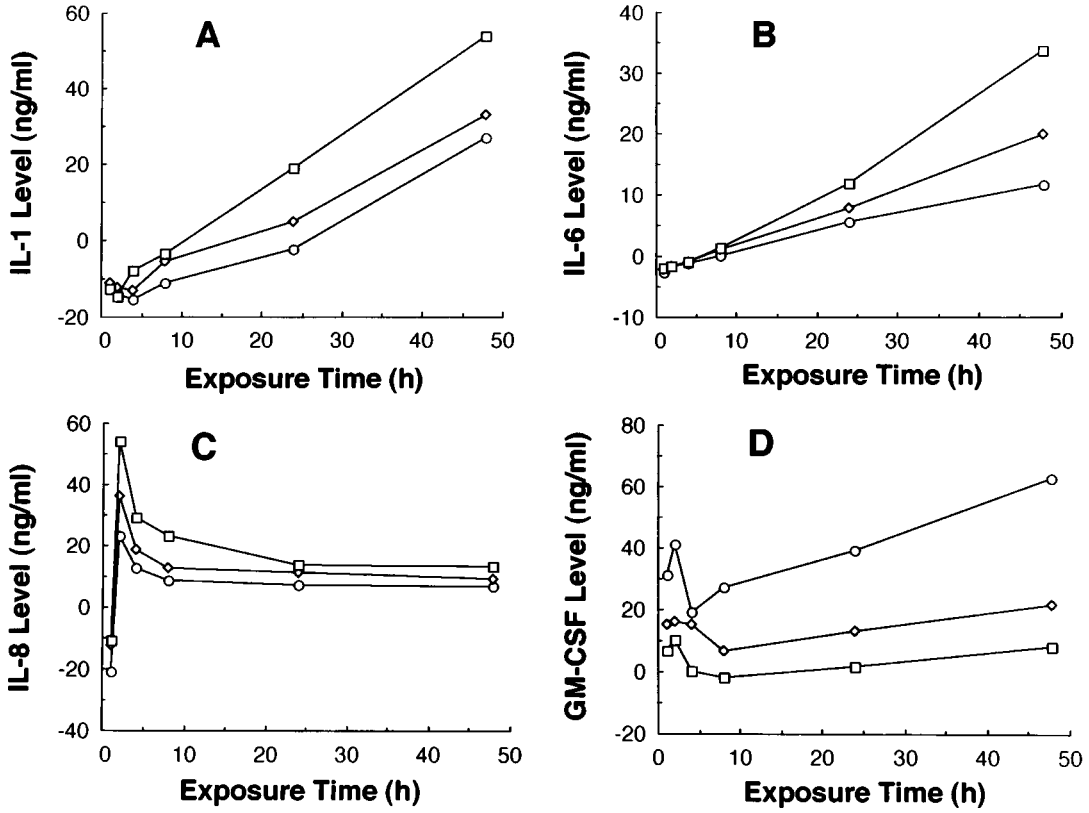

Fig. 3. Levels of IL-1, IL-6, IL-8 and GM-CSF in the medium after the treatment of EC with platinum compounds. Levels of IL-1 (A), IL-6 (B), IL-8 (C) and GM-CSF (D) were measured by the ELISA method as described in "Materials and Methods." Samples were obtained at 1, 2, 4, 8, 24 and 48 h after the exposure of EC to each platinum compound (□ cisplatin, ◇ carboplatin, ○ TRK-710) for 80 min. Each point indicates the difference between cytokine levels secreted by EC treated with platinum compound and by untreated control EC.

with cisplatin, carboplatin and TRK-710. Fig. 2 shows that no morphological changes occurred, regardless of time and drug, compared with the untreated EC.

Secretion of IL-1 Tables I, II and III list the differences of IL-1 secretion between cells treated with platinum compounds and untreated control cells calculated from the levels of IL-1 secreted into the medium by EC after treatment with platinum compounds. The IL-1 levels increased with time, and the IL-1 levels at 48 h after treatment with either cisplatin, carboplatin or TRK-710 were significantly higher than those at 24 h thereafter (P=0.0067, P=0.0027 and P=0.0025, respectively). Fig. 3A shows serial measurements of IL-1 after treatment with platinum compounds. Until 8 h after the treatment, no difference in IL-1 levels between cisplatin, carboplatin and TRK-710 was observed, but the IL-1 levels increased gradually with time thereafter. The IL-1 levels were 53.61±5.68 ng/ml for cisplatin, 32.61±3.36 ng/ml for carboplatin, and 26.75±1.87 ng/ml for TRK-710 after 48 h. Thus, cisplatin induced a significantly greater release of IL-1 from EC than did carboplatin and TRK-710 (P=0.0067 and P=0.0208, respectively). Similarly, cisplatin stimulated EC to release significantly more IL-1 than did carboplatin and TRK-710 after 24 h (P=0.0045 and P=0.0033, respectively).

Secretion of IL-6 Tables I, II and III list the levels of IL-6 secreted into the medium by EC after treatment with platinum compounds. The IL-6 levels increased with time, and the IL-6 levels at 48 h after treatment with either cisplatin, carboplatin or TRK-710 were significantly higher than those at 24 h thereafter (P=0.0077, P=0.0195 and P=0.0014, respectively). Fig. 3B shows the results of serial measurements of IL-6 after treatment with platinum compounds. As in the case of IL-1, until 8 h after the treatment, IL-6 levels showed no difference between the cisplatin, carboplatin and TRK-710 groups. The IL-6 levels were slightly increased at 8 h after the treatment and then gradually increased with time. The IL-6 levels were

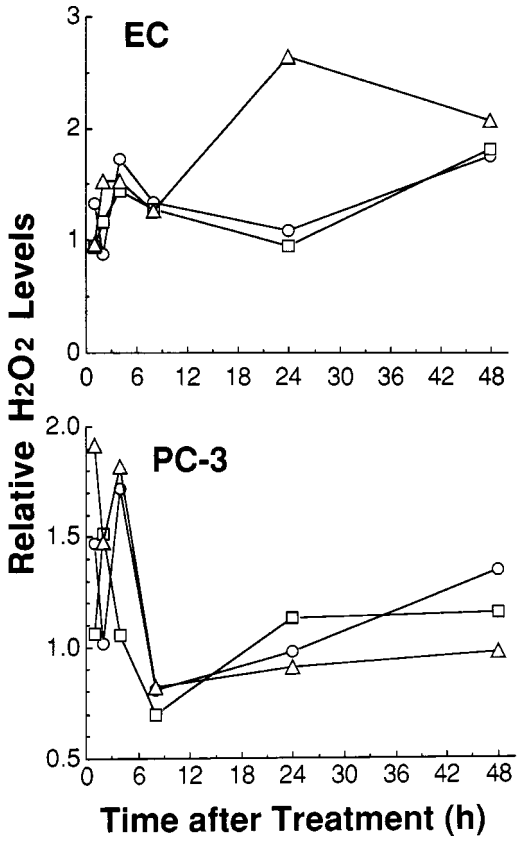

Fig. 4. Intracellular H_2O_2 production after the exposure of EC and PC-3 adenocarcinoma cells to platinum compounds. Intracellular H_2O_2 was detected by measuring the fluorescence of dichlorofluorescein formed from 2′,7′-dichlorofluorescein by H_2O_2. The cells were incubated for 1, 2, 4, 8, 24 and 48 h after the exposure of EC or PC-3 cells to each platinum compound (○ cisplatin, □ carboplatin, △ TRK-710) for 80 min. 2′,7′-Dichlorofluorescein diacetate was added to the medium to give a final concentration of 5 μM 15 min before the end of the incubation. Samples were trypsinized, washed twice with cold PBS, and then subjected to flow cytometry. Each point indicates the ratio of the intracellular H_2O_2 level in EC or PC-3 to that in untreated control EC or PC-3 cells.

33.52±1.41 ng/ml for cisplatin, 19.57±1.10 ng/ml for carboplatin, and 11.34±0.50 ng/ml for TRK-710 after 48 h. Thus, cisplatin induced a significantly greater release of IL-6 from EC than did carboplatin and TRK-710 (P=0.0022 and P=0.0028, respectively). Similarly, cisplatin stimulated EC to release significantly more IL-6 than did carboplatin and TRK-710 after 24 h (P=0.0026 and P=0.0158, respectively). IL-6 was released later and in a smaller amount than IL-1 after the stimulation by platinum compounds.

Secretion of IL-8 Tables I, II and III list the levels of IL-8 secreted into the medium by EC after treatment with platinum compounds. The IL-8 levels increased rapidly after platinum compound stimulation, and the IL-8 levels at 2 h after treatment with either cisplatin, carboplatin or TRK-710 were significantly higher than those at 1 h thereafter (P=0.0003, P=0.0001 and P=0.0007, respectively). After 4 h, the IL-8 levels were significantly lower than those after 2 h (P=0.0011, P=0.0052, P=0.0014). Fig. 3C shows the results of serial measurements of IL-8 after treatment with platinum compounds. The IL-8 release was maximum within 2 h after the treatment with cisplatin, carboplatin and TRK-710, and then the IL-8 levels in the medium decreased rapidly. The IL-8 levels at 2 h after the treatment were 53.87±1.85 ng/ml for cisplatin, 36.15±2.04 ng/ml for carboplatin, and 23.23±1.09 ng/ml for TRK-710. Thus, cisplatin induced a significantly greater release of IL-8 from EC than did carboplatin and TRK-710 (P=0.0006 and P=0.0002, respectively). This significant difference in IL-8 levels between cisplatin and carboplatin or TRK-710 remained until 8 h later. Although IL-8 was rapidly released from EC and decreased to a plateau, the level remained higher than the IL-8 level of the untreated control EC.

Table IV. Levels of Intracellular H_2O_2 (AU)[a)] in PC-3 after Treatment with Cisplatin, Carboplatin and TRK-710

Exposure time (h)	Cisplatin	Carboplatin	TRK-710
1	1.47±0.70	1.06±0.39	1.92±0.33
2	1.02±0.65	1.52±0.82	1.47±0.42
4	1.72±0.38	1.06±0.42	1.82±0.38
8	0.81±0.44	0.70±0.41	0.82±0.33
24	0.98±0.40	1.13±0.36	0.91±0.13
48	1.35±0.62	1.16±0.55	0.98±0.43

Values=mean±standard deviation.
a) Expressed as (level of intracellular H_2O_2 produced in cells treated with platinum compound)/(that in untreated control cells).

Secretion of GM-CSF Tables I, II and III list the levels of GM-CSF secreted into the medium by EC after treatment with platinum compounds. The GM-CSF levels increased for a short period, decreased and then increased again gradually with time, and at 48 h after treatment with either cisplatin, carboplatin or TRK-710 were significantly higher than those at 24 h after treatment (P=0.0132, P=0.0012 and P=0.0042, respectively). Fig. 3D shows the results of serial measurement of GM-CSF after treatment with platinum compounds. TRK-710 induced a greater release of GM-CSF from the EC than did cisplatin or carboplatin after 24 h and 48 h. The GM-CSF levels at 48 h after the treatment were 7.94±1.45 ng/ml for cisplatin, 21.32±0.29 ng/ml for carboplatin and 62.24±0.96 ng/ml

for TRK-710. Thus, TRK-710 induced a significantly greater release of GM-CSF from EC than did cisplatin and carboplatin ($P<0.0001$ and $P=0.0001$, respectively). Similarly, TRK-710 stimulated EC to release significantly more GM-CSF than did cisplatin and carboplatin after 24 h ($P=0.0009$ and $P=0.0031$, respectively).

Effects of platinum compounds on intracellular H_2O_2 production Intracellular levels of H_2O_2 in EC showed a transient increase after treatment with platinum compounds (Tables I, II and III and Fig. 4). However, these increases were not significant. The H_2O_2 levels were 1.09±0.45 AU for cisplatin, 0.95±0.48 AU for carboplatin and 2.64±1.16 AU for TRK-710 after 24 h as compared with the untreated control (1.00). Thus, TRK-710 stimulated intracellular H_2O_2 production more than did cisplatin and carboplatin, but the difference was not significant.

Because the transient increase in intracellular H_2O_2 levels was mild, and because it was difficult to decide if this increase was real, we tested intracellular H_2O_2 production in PC-3 cells. Intracellular H_2O_2 levels in PC-3 cells reached a maximum at 2 h after treatment with carboplatin and 4 h after treatment with cisplatin or TRK-710, and then the fluorescence decreased rapidly to below the control (Table IV). The maximum H_2O_2 levels were 1.72±0.38 AU for cisplatin at 4 h, 1.52±0.82 AU for carboplatin at 2 h and 1.82±0.38 AU for TRK-710 at 4 h, and these levels at 8 h were 0.81±0.44 AU for cisplatin, 0.70±0.41 AU for carboplatin and 0.82±0.33 AU for TRK-710. We failed to find a significant difference between the maximum H_2O_2 levels and the H_2O_2 levels at 8 h later, but both PC-3 cells and EC showed an increase in intracellular H_2O_2 levels, indicating that the platinum compounds may induce transient H_2O_2 production, even in EC.

In either case, platinum compounds induced intracellular H_2O_2 production within a short time after the treatment, and the H_2O_2 was eliminated soon thereafter, except for TRK-710 (after 24 h).

Effects of platinum compounds on mitochondria Because platinum compounds induced intracellular H_2O_2 production, the mitochondria of EC after treatment with platinum compounds were observed morphologically using a confocal laser microscope. The mitochondria showed no changes in Rho uptake within 8 h after exposure of EC to platinum compounds, and no change in appearance was seen (data not shown). However, the EC exposed to TRK-710 showed an increase in numbers of mitochondria and Rho uptake after 24 h and mitochondrial morphological abnormality was seen after 48 h, corresponding to changes of intracellular H_2O_2 levels, whereas the EC exposed to cisplatin and carboplatin showed essentially no change in their numbers or the morphology of their mitochondria compared with the untreated controls (Fig. 5).

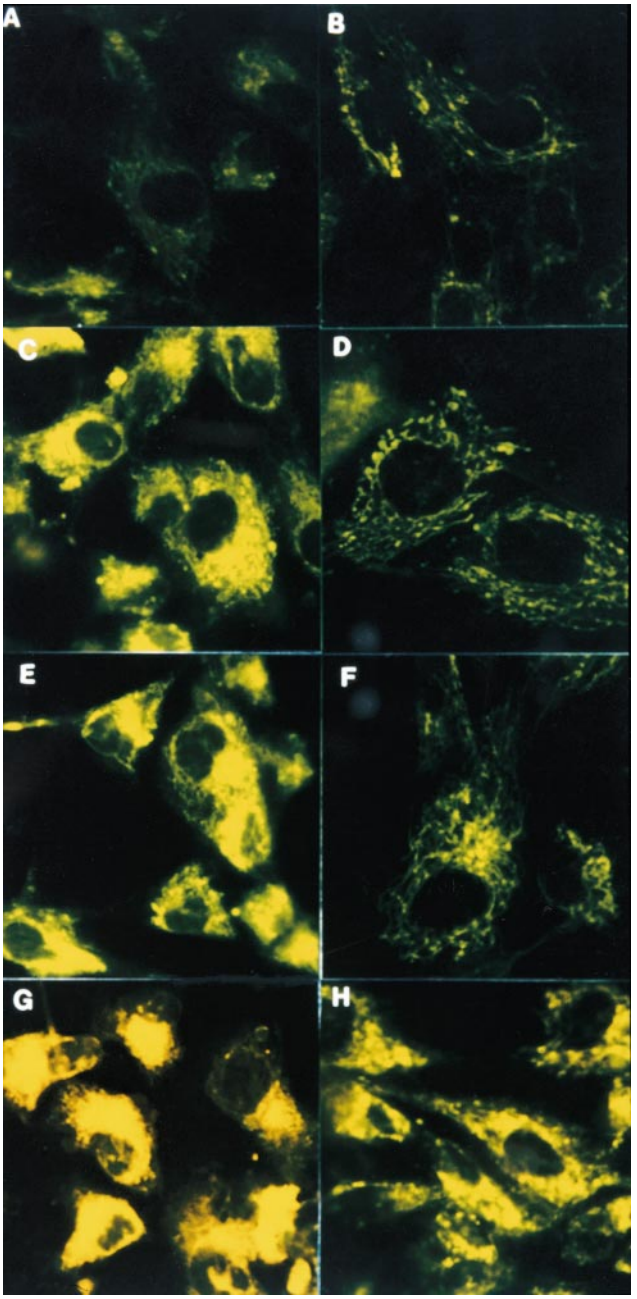

Fig. 5. Effects of platinum compounds on mitochondria of EC. The EC were reincubated for 24 h (in the left panels) and 48 h (in the right panels) after exposure to PBS only (A and B) or to a platinum compound, cisplatin (C and D), carboplatin (E and F) or TRK-710 (G and H), for 80 min. Rho was added to the medium at a final concentration of 10 μg/ml 30 min before the end of the incubation. The mitochondria that were stained yellow with Rho in live EC were observed using a confocal laser microscope. If mitochondria are affected by platinum compounds, Rho uptake would decrease. Conversely, if the potential of the mitochondrial membrane increases, Rho uptake would increase (G and H).

DISCUSSION

We investigated the effects of platinum compounds on cytokine production in human umbilical vein EC and found that cisplatin most prominently induced the release of IL-1 and IL-6 from the EC and that TRK-710 induced the release of significantly more GM-CSF than cisplatin and carboplatin.

IL-8 was produced immediately after the exposure of the EC to platinum compounds, but subsequently decreased, indicating that the EC may be damaged by platinum compounds. But the action of IL-8 was transient and insufficient, because morphological changes were not observed within 48 h after the exposure of the EC to the platinum compounds. As IL-8 mediates inflammation and induces H_2O_2 production,[15, 39] and as its secretion and H_2O_2 production occurred simultaneously, we suppose that IL-8 may have some relation to H_2O_2 generation. The ability to produce H_2O_2 or IL-8 was different between the platinum compounds: cisplatin most prominently induced IL-8 secretion, and TRK-710 most prominently induced H_2O_2 production. Thus, the effects of platinum compounds on intracellular H_2O_2 production and IL-8 secretion differed. As we did not measure the intracellular IL-8 levels, the relationship between intracellular H_2O_2 production and IL-8 levels is unknown. Furthermore, as we failed to clarify if the platinum compounds directly induced IL-8 release or H_2O_2 production, further experiments are required to determine the relationship.

The release of IL-1 increased from 8 h after the exposure of EC to platinum compounds, indicating that a time-lag occurs between the stimulation by the platinum compounds and the release of IL-1. IL-1 is known to induce and to cause release of superoxide dismutase (SOD) from mitochondria of the EC.[40] SOD catalyzes the dismutation of O_2 to H_2O_2.[41] As described above, we found that platinum compounds had the ability to induce intracellular H_2O_2 production, and that intracellular H_2O_2 production and IL-8 release occur simultaneously in the EC. However, IL-1 was secreted from the EC after intracellular H_2O_2 production, even though it has the ability to induce Mn-SOD and Cu,Zn-SOD. The times at which IL-1 acted, and at which SOD was induced are still unclear, because we did not measure the intracellular IL-1 and SOD levels. On the other hand, a difference between the platinum compounds in the ability to induce IL-1 release from the EC was observed. However, the levels of intracellular H_2O_2 were rather similar, indicating that other factors may be associated with H_2O_2 production and IL-1 release. On the basis of these results, we considered that intracellular H_2O_2 production induced by platinum compounds might induce IL-1 release after the elimination of the intracellular H_2O_2. Nakata *et al*.[40] reported that Mn-SOD, which is localized in the mitochondria, increases after IL-1 stimulation. We found that the mitochondria did not change morphologically within 8 h after stimulation by platinum compounds. This result indicates that Mn-SOD may act without damaging mitochondria, or that Mn-SOD may not have any role in the mitochondrial damage.

The time-lag between the release of IL-6 and the stimulation by platinum compounds was more prolonged. IL-6 is also reported to have an ability to induce SOD, but its ability is very low compared with that of IL-1.[42] On the other hand, IL-1 stimulates intracellular IL-6 production, and IL-6 is released from EC 8 h after IL-1 stimulation,[9, 10] indicating that IL-6 production may take 16 h after the stimulation by platinum compounds. Therefore, from our results and these reports, we consider that the IL-6 release may be induced by IL-1 stimulation. The association of IL-1 levels with IL-6 levels may be consistent with our interpretation that IL-1 stimulates intracellular IL-6 production.

Thus, the series of events after treatment with platinum compounds may be interpreted as the result of inflammation initiated by the treatment. However, GM-CSF was released only by TRK-710 stimulation. Why TRK-710 has the ability to induce GM-CSF release from EC is still unknown. However, unlike cisplatin and carboplatin, TRK-710 has a [(5S)-3-acetyl-5-methyl-2,4(3H,5H)-furandionate-O^3,O^4] ligand, which can induce GM-CSF in the dog (unpublished data). Thus, GM-CSF may be induced via release of the ligand from TRK-710. On the other hand, TRK-710 showed prominent H_2O_2 production and mitochondrial abnormality simultaneously, which may be associated with GM-CSF secretion. Further experiments are required to clarify this relationship.

Thus, platinum compounds act on EC to release IL-1, IL-6 and GM-CSF. The platinum compounds, except for TRK-710, have severe hematological side effects. These side effects may be due to the direct effects of cisplatin and carboplatin on progenitor cells in the bone marrow.[43] However, generally, platinum compounds are ineffective against acute myelogenous leukemia, in spite of their severe hematological toxicity.[44] IL-1 has an important role as a regulator of hematopoiesis by inducing a variety of hematopoietically active cytokines and by synergizing with these cytokines to amplify the hematopoietic response.[45] Similarly, IL-6 affects the T cells, myeloid cell differentiation, regulation of acute-phase reactants, and the development and maturation of megakaryocytes.[46] Therefore, both IL-1 and IL-6 were investigated for the ability to overcome myeloid suppression induced by anticancer agents. Although it is not known whether IL-1 and IL-6 released from EC treated with platinum compounds stimulate hematopoiesis *in vivo*, we suppose, from reports[47, 48] of clinical phase studies that the median time to recovery of neutrophils is almost 12 days, that both IL-1 and IL-6 may participate in hematopoiesis by inducing a

variety of hematopoietically active cytokines and by synergizing with these cytokines to amplify the hematopoietic response.[45] These reports and our results indicate that IL-1 and IL-6 may also act as inducers of hematopoiesis *in vivo*. However, further *in vivo* studies are necessary to confirm this.

TRK-710 also induced the release of IL-1 and IL-6, though at low levels compared with cisplatin and carboplatin. Moreover, TRK-710 released significantly more GM-CSF than cisplatin and carboplatin, thus maintaining the peripheral leukocyte numbers at normal levels by releasing mature leukocytes from the bone marrow.

The vein EC of cancer patients are obviously senescent compared with umbilical vein EC. Whether the vein EC of cancer patients can release cytokines in the same way as umbilical vein EC is still unclear. However, as Engerman *et al*.[49] have reported that EC in the normal adult male are metabolically active, although they are quiescent because of the low turnover, our findings may provide a new insight into hematological disorders induced by anticancer agents and into the prevention of hematological toxicity of such agents.

In conclusion, platinum compounds, such as cisplatin and carboplatin, induced cytokine release from EC through a series of inflammation-related events: H_2O_2 production and IL-8 release, decrease in mitochondrial potential, and IL-1 secretion followed by IL-6 release. TRK-710 increased mitochondrial potential and induced GM-CSF secretion from EC. IL-1 and IL-6 and GM-CSF may participate in hematopoiesis to counteract the hematological side effects induced by chemotherapy.

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