Enhancement of Bone Marrow CD41+ Megakaryocytes as well as Modulation of TNF-α, IL-12, and IL-5 by a Novel Small Molecule, UTL-5g, in Mice Treated with Cisplatin

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Abstract

UTL-5g is a novel small-molecule chemoprotective agent against cisplatin-induced host cytotoxicity. Recent studies showed that UTL-5g increased the blood platelet count, which was reduced in cisplatin-treated mice. In order to have a better understanding about the mechanism of action by which UTL-5g promotes platelet production, BDF1 mice were treated with or without UTL-5g after cisplatin treatment; several hematological analyses were conducted. The results showed that treatment of mice with UTL-5g alone by either *i.p.* injection or oral gavage markedly increased platelet counts. UTL-5g also restored bone marrow cell counts that were reduced by cisplatin treatment. Flow cytometric analysis showed that bone marrow CD41+ megakaryocytic cells were significantly increased in animals pretreated with UTL-5g before cisplatin. Measurement of secreted cytokines showed that pretreatment of UTL-5g lowered blood levels of both TNF-α and IL-12 (p70) elevated by cisplatin treatment. On the other hand, pretreatment of UTL-5g raised blood levels of IL-5 that were lowered by cisplatin treatment. The results also showed that 60 mg/kg is the optimal dose of UTL-5g by oral route for the protection of bone marrow cells, CD41+ megakaryocytes, and platelets. In conclusion, this study suggests that UTL-5g (by *i.p.* injection or oral gavage) enhances the production of platelets by either protecting or increasing bone marrow CD41+ megakaryocytic cells at least in part; UTL-5g also modulates TNF-α, IL-12 (p70), and IL-5. Taken together, these observed effects of UTL-5g on megakaryocytes and cytokines play important roles in the chemoprotective properties of UTL-5g.

Keywords: chemoprotective agent, cisplatin, platelets, CD41+ megakaryocytes, bone marrow cells.

1. Introduction

Cisplatin is a widely used cytotoxic agent with activity against many human cancers including testicular, ovarian, gastrointestinal, head and neck, and lung. However, cisplatin is also associated with substantial undesirable side effects, a major one being nephrotoxicity, which evolves slowly but predictably after initial and repeated exposure [1, 2]. Another side effect associated with cisplatin is hepatotoxicity, which is the dose-limiting toxicity. Hepatotoxicity occurs when cisplatin is administered at high doses [3, 4] and is often associated with the elevation of alanine aminotransferase (ALT), aspartate aminotransferase (AST), nitric oxide (NO) and calcium levels in the blood [5]. Additionally, about 25 to 30 percent of patients undergoing cisplatin treatment may also develop myelosuppression [6], in which the numbers of platelets and white blood cells (leukocytes) are severely reduced following 2 to 3 weeks of treatment. The loss of platelets (thrombocytopenia) and leukocytes (leukopenia) are more pronounced when higher doses of cisplatin are given [6].

To overcome these toxic side effects, many natural and synthetic compounds have been investigated and shown to be chemoprotective [7, 8]. Yet, there is only one FDA approved drug being widely used as a broad-spectrum chemoprotector, amifostine, which is a sulfur-containing agent that reduces side effects from various chemotherapy and radiotherapy regimens [9, 10]. Unfortunately, amifostine is also associated with a number of side effects [10]. In addition, amifostine may hamper the therapeutic effect by rapid chemical quenching of cisplatin to form an inactive platinum–thiol conjugate species [11]. Therefore, the search for a significantly improved chemoprotective agent against cisplatin-induced side effects continues.

Among the earliest molecular effects caused by cisplatin are the activation of the mitogen-activated protein kinase (MAPK) signaling cascade and molecular pathway responses to stress. Inflammatory cascades are important determinants of the degree of organ failure induced by cisplatin, which are often associated with abnormal induction of TNF-α in both affected tissues and blood. Recent studies showed that TNF-α plays a crucial role in the pathogenesis of cisplatin-induced nephrotoxicity [12-15]. Zhang et al. showed that the production of TNF-α by renal parenchymal cells is responsible for cisplatin-induced nephrotoxicity [16]. Still others showed that chemoprotection by phenolic antioxidants is through the control of TNF-α induction, indicating that modulation of TNF-α is a potential mechanism to achieve chemoprotection [17]. Excessive amounts of TNF-α have also been shown to cause severe injury and damage to normal tissues and bone marrow hematopoietic stem cells resulting in thrombocytopenia and leucopenia [18, 19].

We have shown that a small-molecule, UTL-5g, lowered TNF-α levels elevated by cisplatin and reduced cisplatin-induced hepatotoxicity and nephrotoxicity [20]. We also showed that UTL-5g could reduce the acute liver damage caused by local irradiation [21]. In addition, we showed that UTL-5g can markedly increase blood platelet counts in cisplatin-treated mice [20] indicating that UTL-5g might be effective in suppressing cisplatin-induced thrombocytopenia. Therefore, UTL-5g is a potential chemoprotective agent worth further investigation; this work was set up to gain a better understanding about the potential mechanism of action by which UTL-5g protects platelet production.

Platelet glycoprotein IIb (CD41 antigen) is expressed on mature platelets and megakaryocytes as well as their progenitor cells in bone marrow [22-24]. CD41 associates with CD61 (platelet glycoprotein IIIa) in a complex to form the integrin GPIIb/IIIa, which plays a major role in platelet function [25]. Since CD41 is thought to be highly specific to cells in the megakaryocytic/platelet lineage, we set out to investigate the effect of UTL-5g on the number of CD41 cells in mouse bone marrow following cisplatin and with and without UTL-5g pretreatments. We now report that the increase of blood platelet counts by UTL-5g may be through either protecting or enhancing the number of

bone marrow CD41+ cells in mouse at least in part.

2. Materials and Methods

2.1 Chemicals reagents and media

UTL-5g (Lot #1182-MEM-3D, Purity >99%) was synthesized by Kalexsyn (Kalamazoo, MI). Alpha-MEM was purchased from MediaTech (Manassas, VA). Bovine calf serum was purchased from HyClone Laboratories (Logan, Utah, US). Bio-Plex Pro™ Mouse Cytokine Th1/Th2 assay for IL-2, IL-4, IL-5, IL-10, IL-12 (p70), GM-CSF, IFN-γ, and TNF-α (M60-00003J7) and Bio-Plex Pro™ TGF-β 3-plex assay for TGF-β 1,2 and 3 (171-W4001M) were purchased from BioRad (Hercules, CA). Allophycocyanin conjugated anti-murine CD41 antibody (APC-anti-CD41) was obtained from BioLegend (San Diego, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

2.2 Animals

BDF1 female mice (8-10 weeks, ~20 g each) were purchased from Charles River (Wilmington, MA). All animal studies were in full compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines.

2.3 Preparation of UTL-5g and cisplatin

For *i.p.* injection (60 mg/kg), UTL-5g was dissolved in equal volume of dimethyl sulfoxide (DMSO) and cremophor/propylene glycol (60/40 v/v). The solution was then diluted with saline (1:9 v/v) to the final concentration 4.8 mg/mL. For oral gavage (60, 120 and 240 mg/kg), UTL-5g was dispersed in OraPlus® (Paddock Laboratories), by using a mortar and pestle to make suspensions of UTL-5g at 4.8, 9.6, and 19.2 mg/mL, respectively. Cisplatin was dissolved directly in saline to 0.2 mg/mL.

2.4 Animal Studies

BDF1 female mice were randomly divided into six groups (5 mice per group) and each treated as follows: (Group A) medium (saline) followed by medium (saline) 30 min later; (Group B) medium (saline) followed by cisplatin (2.5 mg/kg, *i.v.*) 30 min later; (Group C) UTL-5g (60 mg/kg, *i.p.*) followed by cisplatin (2.5 mg/kg, *i.v.*) 30 min later; (Group D) UTL-5g (60 mg/kg, oral) followed by cisplatin (2.5 mg/kg, *i.v.*) 30 min later; (Group E) UTL-5g (120 mg/kg, oral) followed by cisplatin (2.5 mg/kg, *i.v.*) 30 min later and (Group F) UTL-5g (240 mg/kg, oral) followed by cisplatin (2.5 mg/kg, *i.v.*) 30 min later. UTL-5g administration volume (either *i.p.* injection or oral gavage) was 0.25 mL/mouse daily for 5 days. Cisplatin was prepared as a single injection. On day 6, blood from each animal was obtained by cardiac puncture in a heparinized syringe. White blood cells and platelet counts from each blood sample were carried out using a Leukochek™ test kit (Biomedical Polymers, Gardner, MA) diluting systems and a hemocytometer. Bone marrow cell count was performed for each animal and bone marrow cells were processed for CD41+ megakaryocytes (Mk) as described below.

2.5 Preparation for CD41+ megakaryocytes and cytokine analysis

Bone marrow cells ($1x10^6$ cells) were fixed in 4% paraformaldehyde diluted with staining buffer (1 x PBS, 0.1% sodium azide, 0.5% BSA). After fixing, cells were washed once in staining buffer then incubated with anti-murine CD41 antibody conjugated with allophycocyanin (APC) (BioLegend, San Diego, CA). After incubation at room temperature for 30 min, cells were washed with staining buffer followed by PBS. Thereafter, cells were fixed with 3 mL of ice cold ethanol added dropwise while vortexing. All samples were then incubated overnight at 4 °C in the dark. On the following day, cells were pelleted, ethanol was aspirated, and pellets washed once with PBS. After washing, cells were incubated for 2 hr at 4 °C with propidium iodide (PI) staining solution (eBioscience, San Diego, CA) to stain DNA then 100,000 PI+ events for analysis were acquired using an LSRII flow cytometer (BD Biosciences, San Jose, CA) and results were analyzed using DIVA software (v 6.1.3).

The levels of blood cytokines were analyzed using Bio-Plex Pro™ Th12/Th2 mouse cytokine assay (BioRad) according to the manufacturer's instruction. Plasma samples (50 µL of sample

dilution/well) from each group were analyzed using the Bio-plex 200 (BioRad) for both TNF-α and interleukin-5 (IL-5). Samples for TGF-α (1, 2 and 3) determination were measured separately using the Bio-Plex Pro™ TGF-β 3-plex assay, following the manufacturer's directions(BioRad). Cytokine concentrations were determined using Bio-plex Manager Software (v 5.0) (BioRad).

3. Results and Discussion

3.1 Effect of UTL-5g on platelet count

As shown in Fig. 1, the pretreatment of UTL-5g by either *i.p.* injection or oral gavage significantly increased the platelet counts as compared to animals treated with cisplatin only (group B). Both groups C (60 mg/kg, *i.p.* injection) and D (60 mg/kg oral gavage) showed significant increases of platelet counts (28% and 30% respectively). Groups E (120 mg/kg, oral gavage) and F (240 mg/kg, oral gavage) also showed a statistically significant increase in the platelet counts as compared to Group B (34% and 46% respectively). This observation is consistent with what we reported in the previous studies where we showed that the pretreatment of UTL-5g increased platelet counts whether the animals were treated with cisplatin (30 min later) or not [20]. These results also showed that the optimal oral dose for UTL-5g under current experimental conditions appears to be in the range of 60 to 120 mg/kg.

3.2 Effect of UTL-5g on bone marrow cells

The regimen used for cisplatin treatment (2.5 mg/kg x 5) was sufficient to cause significant bone marrow injury. As shown in Table 1, animals treated with cisplatin (Group B) have much lower bone marrow cell counts as compared to those in the control group (Group A) (6.84 vs. 12.1 x 10^6). In animals pretreated with UTL-5g (whether by *i.p.* or oral administration), the reduced bone marrow cell counts were partially recovered, indicating that UTL-5g protected overall bone marrow cells from cisplatin-induced injury. The protection by UTL-5g was significantly increased with *i.p.* 60 mg/kg (Group C) (8.83 x 10^6) and oral 60 mg or 120 mg/kg (9.66 and 9.32 x 10^6 respectively)

compared to Group B (6.84 x 10^6) wherein animals were treated with cisplatin only. However, at 240 mg/kg of UTL-5g (Group F), the recovery of bone marrow cells was slightly less than those treated with lower doses of UTL-5g indicating that UTL-5g at 240 mg/kg is above the optimal dose. In addition, our results indicate that, under current experimental conditions, the hematological effects of UTL-5g by oral administration at 60 mg/kg, is similar to that by *i.p.* injection and the optimal oral dose is around 60 mg/kg with respect to bone marrow protection.
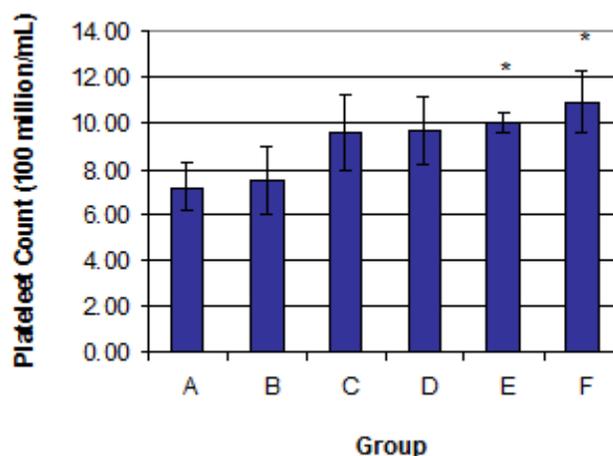

Figure 1. **Comparing the effect of UTL-5g pretreatment on platelet count by *i.p.* injection and by oral gavage.**
(**Group A**) Control (saline); (**Group B**) cisplatin (2.5 mg/kg); (**Group C**) UTL-5g (60 mg/kg, *i.p.*) followed by cisplatin (2.5 mg/kg); (**Group D**) UTL-5g (60 mg/kg, oral) followed by cisplatin (2.5 mg/kg); (**Group E**) UTL-5g (120 mg/kg, oral) followed by cisplatin (2.5 mg/kg); (**Group F**) UTL-5g (240 mg/kg, oral) followed by cisplatin (2.5 mg/kg). * P<0.05 for Group B vs Group E and Group B vs Group F.

### 3.3 Effect of UTL-5g on bone marrow CD41+ cells

In an attempt to better understand how the platelet count is increased by UTL-5g, CD41 expression on MKs was performed using flow cytometry. . Bone marrow c were obtained from mice treated with different doses of UTL-5g were analyzed for CD41 expression. Intact cells were

first gated using forward and side scatter properties (Fig. 2A). These cells were subsequently gated using PI staining (Fig. 2B), and PI+ cells were then analyzed for CD41 expression (Fig. 2C). We observed higher levels of CD41 positive cells from animals pretreated with *i.p.* UTL-5g at 60 mg/kg (1.36%) and oral UTL-5g at 60 and 120 mg/kg (1.82 and 1.70% respectively) than did cells from untreated animals (1.08%) or those treated with cisplatin alone (1.28%); oral UTL-5g at 240 mg/kg appeared to be sub-optimal (Table 1). When normalized for bone marrow cellularity, it became clear that the levels of CD41+ megakaryocytes were significantly higher for all groups pretreated with UTL-5g, whether by oral or *i.p.* administration. Furthermore, under current experimental conditions, using the daily x 5 schedule, oral administration of UTL-5g at 60 or 120 mg/kg appears to be optimal doses for increasing levels of CD41+ megakaryocytes. This dose-bioactivity relationship is similar to that observed in bone marrow protection by UTL-5g (Table 1).
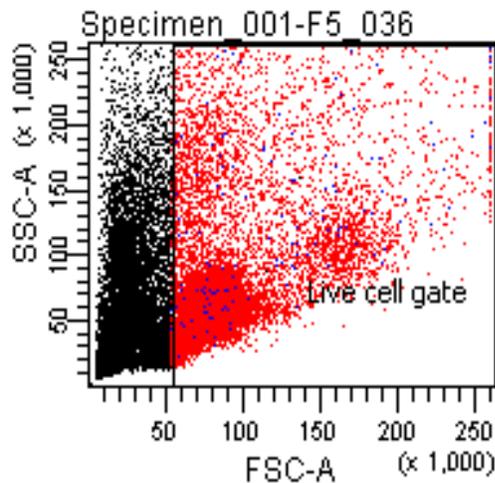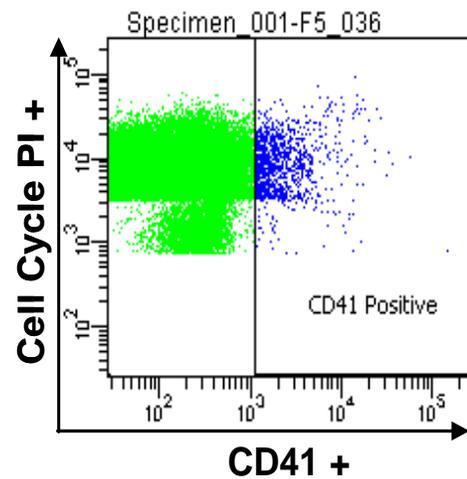
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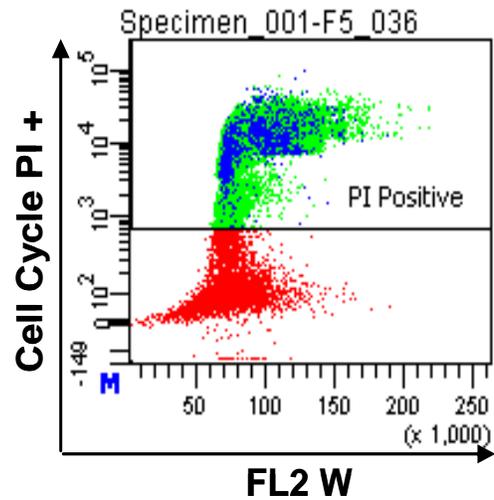
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Figure 2. Gating strategy for CD41 expression on megakaryocytes.
(A) Intact cells were gated to exclude debris and dead cells. (B) These cells were subsequently gated using PI staining. (C) PI+ cells were analyzed for CD41 expression.

(A)

Table 1. CD41 Expressing Megakaryocytic Cells Normalized for Bone Marrow Cellularity[a]

Group	A	B	C	D	E	F
	Control	Cisplatin	UTL-5g 60 mg/kg i.p. + cisplatin	UTL-5g 60 mg/kg oral + cisplatin	UTL-5g 120 mg/kg oral. + cisplatin	UTL-5g 240 mg/kg oral + cisplatin
Bone marrow cell count (10^6)	12.1±1.13	6.84±0.43	8.83±0.60	9.66±0.40	9.32±1.16	7.59±0.39
%CD41+ Mk in Cell Cycle PI+ gate	1.08	1.28	1.36	1.82	1.70	1.34
CD41+ Mk normalized for bone marrow cellularity (10^4)	13.1±1.22	8.75±0.55	12.0±0.82	17.6±0.73	15.8±1.97	10.2±0.52
Ratio of CD41+ Mk as compared to Group A	1	0.67	0.92	1.34	1.21	0.78

[a] Each number represents average ± standard error of 4 mice (groups A, D, and E) and 5 mice (groups B, C, F).

3.4 Effects of UTL-5g on blood levels of TNF-α, IL-12 (p70), and IL-5

Cytokine assay results (Table 2) show that blood levels of both TNF-α and IL-12 (p70) were significantly elevated by cisplatin while that of IL-5 was significantly lowered by cisplatin (group B) as compared with control (group A). TNF-α was increased by 58%, IL-12 (p70) was increased by 257%, and IL-5 was decreased by 42%. For groups D and E, mice were pretreated with oral UTL-5g at 60 and 120 mg/kg, elevated TNF-α blood levels were significantly lowered, which is consistent with our previous observations [20]. It is not clear why the other doses, *i.p.* 60 mg/kg and oral 240 mg/kg (groups C and F) slightly increased TNF-α levels.

Table 2. Effect of UTL-5g on Blood Levels of Cytokines

Group[a]	A	B	C	D	E	F
	Control	Cisplatin	UTL-5g 60 mg/kg i.p. + cisplatin	UTL-5g 60 mg/kg oral + cisplatin	UTL-5g 120 mg/kg oral. + cisplatin	UTL-5g 240 mg/kg oral + cisplatin
TNF-α (pg/mL)	193±33.5	306±31.1	346±53.8	186±11.1	269±42.3	366±23.6
IL-12 (p70) (pg/mL)	8.9±6.6	31.8±2.6	13.9±5.0	14.9±0.9	23.0±7.4	29.2±4.4
IL-5 (pg/mL)	21.9±5.4	12.8±1.6	74.0±50.0	170±159	16.4±4.5	85.7±45.8

[a] Each number represents average ± standard error of 5 mice (groups B) and 5 mice (all other groups).

IL-12 is a biomarker of the disease activity in polyneuropathy, organomegaly, endocrinopathy, M-protein, and skin changes syndrome (POEMS). The overproduction of IL-12 could contribute to the peripheral nerve demyelination characteristic in POEMS [26]. Elevated levels of IL-12 (p70) is associated with optic neuropathy in human primary open angle glaucoma [27]. Table 2 shows that UTL-5g significantly lowered elevated levels of IL-12 (p70) in blood, whether it was administered orally or by *i.p.* injection; UTL-5g at 60 mg/kg (either by *i.p.* or oral administration) appeared to be the optimal dose for lowering IL-12 (p70). These results indicate that UTL-5g may also be a potential agent for treating neuropathy related disorders.

IL-5 is an important cytokine that regulates B cell growth, immunoglobulin secretion, eosinophil activation and survival [28]. Levels of IL-5 were markedly lowered (58%) by cisplatin treatment, indicating that cisplatin may contribute to some negative biological and immunological side effects; the immunosuppressive effect of cisplatin has been reported in previous studies

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[29, 30]. Our finding suggests that reduction of IL-5 may contribute, in part to the immunotoxic effect of cisplatin. As shown in Table 2, the lowering of IL-5 levels by cisplatin was reversed by UTL-5g whether it was administered orally or by *i.p.* injection.

Overall, oral UTL-5g at 60 mg/kg appeared to be the optimal dose in lowering blood levels of TNF-α/IL-12 (p70) and in elevating IL-5. No significant changes were observed in this study for other cytokines tested, including TGF-β (1, 2, and 3), GM-CSF, IL-2, IL-4, and IFN-γ.

In summary, this study suggests that UTL-5g (by *i.p.* injection or oral gavage) enhances the production of platelets, at least in part, by either protecting or increasing bone marrow CD41+ megakaryocytic cells adversely affected by cisplatin; UTL-5g also modulates blood levels of TNF-α, IL-12 (p70), and IL-5 abnormally regulated by cisplatin. Taken together, these observed beneficial effects of UTL-5g on megakaryocytes and cytokines play important roles in the chemoprotective properties of UTL-5g.

Acknowledgement

Abbreviation: APC, allophycocyanin; BSA, bovine serum albumin; IL-5, interleukin-5; IL-12, interleukin-12; Mk, megakaryocytes; PBS, phosphate-buffer saline; PI, propidium iodide; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha.

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