

Granulocyte Colony-Stimulating Factor Enhances the Anticancer Effects of Cisplatin against Lung Cancer by Promoting Angiogenesis

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Abstract

The G-CSF is used as a therapeutic drug of the febrile neutropenia in lung cancer chemotherapy, however, there were few reports that showed the effects of combination effects of G-CSF and anticancer drugs against lung cancer. In the present study, we investigated the effects of G-CSF and the combination effects of G-CSF and cisplatin on lung cancer growth. We investigated the effect of G-CSF against the LL-2 and KLN-205 cells by MTT assay and tried to detect the G-CSF receptor by RT-PCR. Next, to analyze the G-CSF effects *in vivo*, we transplanted the LL-2 into C57BL/6 mice, intraperitoneally administered G-CSF (30 micro/kg/day) with or without cisplatin (5 mg/kg), measured the tumor size and analyzed pathologically by HE and immunostaining. *In vitro* analyses, G-CSF showed no effects in LL-2 and KLN-205 cells, and RT-PCR revealed no G-CSF receptor mRNA. *In vivo* analyses, G-CSF alone did not significantly suppress tumor growth. However, concurrent G-CSF administration with cisplatin significantly enhanced the tumor suppressing effect of cisplatin in early stage of tumor growth. The analysis data of vWF immunostaining indicated that the neovascularization in the peripheral region of the tumors was more enhanced in G-CSF treatment mice. ELISA assay revealed that G-CSF did not influence the serum concentration of TNF-alpha and IL-12 in tumor-bearing mice. This study suggests that concurrent (combination) administration of cisplatin with G-CSF is a safe and effective method for enhancing anticancer effects and reducing chemotherapeutic agent-induced myelosuppression.

Keywords

Granulocyte Colony-Stimulating Factor, Lung Cancer, G-CSF Receptor, Angiogenesis, Anticancer Agent, Cisplatin, TNF-Alpha, IL-12

1. Introduction

Cytokine granulocyte colony-stimulating factor (G-CSF) is a hematopoietic growth factor which enhances the proliferation and differentiation of neutrophil precursor cells [1]. In addition, it stimulates a variety of responses in peripheral blood neutrophils including phagocytosis, superoxide production and chemotaxis [2]. G-CSF is clinically used to enhance the recovery of the number and function of neutrophils during chemotherapy-induced neutropenia in various hematogenous and nonhematogenous malignant and nonmalignant diseases [3] [4] [5] [6] [7]. However, the results of studies on G-CSF-induced tumor growth are controversial. Recently, some studies reported that G-CSF stimulates the growth of tumor cells such as colon cancer cells [8], small lung cancer cells [9], skin carcinoma cells and astrocytoma cells [10] [11]. In contrast, Brandstetter *et al.* reported that G-CSF does not exhibit any effect on the proliferation of ovarian carcinoma cell lines or tumor samples despite presence of the G-CSF receptor in the tested cell lines and biopsies [12]. Moreover, G-CSF inhibits tumor growth in a B16-BL6 melanoma cell transplanted mice model [13]. However, none of these studies were conducted using concurrent administration with anticancer agents. G-CSF as an adjuvant with cytotoxic chemotherapeutic agent in cancer chemotherapy is very important to clarify its effects on tumor growth in combination with anticancer agents. Thus, to further elucidate the role of G-CSF in tumor growth, especially in combination with anticancer drugs, we used one of the most useful clinical anticancer drug cisplatin (CDDP) [14] [15] [16] [17], whose most serious side effect is bone marrow suppression [18] [19] [20], concurrently administered with G-CSF in a transplanted lung cancer cells mouse model, and the possible mechanism responsible for the effects of G-CSF on tumor growth was studied *in vitro* and *in vivo*.

2. Materials and Methods

2.1. *In Vitro* Effects of G-CSF on Tumor Cell Proliferation

The effect of G-CSF on tumor growth *in vitro* was determined by 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium (MTT) assay as previously described [21] [22] [23] [24]. Two mouse non-small lung cancer cell lines, Lewis lung cancer cell line (LL-2) and KLN-205 (Japanese Collection of Research Bioresources Cell Bank, Ibaraki, Japan) were grown in DMEM medium with 10% fetal bovine serum (FBS). One hundred microliters of 1.0×10^5 cells/ml cells per well were seeded in 96-multiwell plates and precultured for 24 hours. Thereafter, the cells were stimulated without (control) or with various concentrations of G-CSF sup-

plements with 2% FBS for 48 hours. Each experiment was performed in triplicates, and the results were expressed as the relative viability percentage as follows:

Relative viability percentage = (mean OD560nm for the G-CSF test/without G-CSF control) \times 100%.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR) for G-CSF receptor mRNA detection.

Bone marrow cells isolated from a normal six weeks-old male C57BL/6 mouse as previously described [25] were used as a positive control for G-CSF receptor mRNA. Total RNA was extracted from cultured LL-2 and KLN-205 cells as well as from unseparated bone marrow cells using Isogene (Nippongene, Chiyodaku, Japan), and 3 microg of the total RNA was subjected to a reverse transcription reaction using a First Strand cDNA Synthesis kit (Amersham Biosciences Corp., Piscataway, NJ) according to the manufacture's instructions. After reverse transcription, the cDNA was subjected to the following PCR reaction: (a) the beta-actin-specific 967 bp fragment was detected by PCR (25 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min) using the primers 5'-GTGACGAGGCCAGAGCAAGAG-3' (sense) and 5'-AGGGGCCGGACTCATCGTACTC-3' (anti-sense); (b) the G-CSF receptor-specific 567 bp fragment was detected by PCR (38 cycles at 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min) with the primers 5'-CCCCTCAAACCTATCCTGCCTC-3' (sense) and 5'-TCCAGGCAGAGATCAGCGAATG-3' (anti-sense) [26]. The negative PCR reactions control contained water instead of DNA template. To eliminate artifacts, RT-PCR was performed at least three times.

2.2. *In Vivo* Evaluation of the Effects of G-CSF on Tumor Growth

Seven week-old male C57BL/6 mice were purchased from CLEA Japan (Meguroku, Japan). LL-2 cancer cells were grown in culture, harvested and subcutaneously injected as a suspension (1.0×10^5 cells in 0.1 ml of serum-free medium) into the C57BL/6 mice in the proximal dorsal midline. The mice were randomized into 4 groups (ten mice in each group), group 1) saline control, 2) G-CSF alone, 3) CDDP alone and 4) CDDP plus G-CSF group. The mice were injected 5 mg/kg CDDP intraperitoneally 2 hours after tumor cell transplantation and then, were given 5 mg/kg CDDP intraperitoneally each week. Two hours after CDDP or saline injection, the mice were given 30 micro/kg G-CSF or the same volume of saline intraperitoneally each day, and 21 days after tumor cell transplantation they were sacrificed and the tumors were removed. To investigate the mechanism responsible for G-CSF activity on tumor growth, twelve mice were transplanted LL-2 tumor cells (1.0×10^5 cells/0.1 ml) and divided equally two groups. One group was injected 30 micro/kg G-CSF and the other was injected the same volume of saline each day and 7 days after treatment they were sacrificed and the tumors were removed. Excised tumor specimens were fixed in 10% buffered formalin, and tumor size was measured along the longest superficial

diameter (a) and smallest superficial diameter (b), and tumor volume expressed as: $a \times b^2 \times 0.52$ (mm³).

2.3. Evaluation of Microvessel Density

Sections 4 micro m thick which were taken 7 days after tumor cell transplantation were de-paraffinized and incubated with anti-von Willebrand factor (vWF) rabbit antibody (DAKO Cytomation Co. Ltd., Kyoto, Japan). Immunolabelling was performed using an avidin-biotin-peroxidase technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) followed by counterstaining with hematoxylin, and negative control slides were obtained by omitting the primary antibody. Microvessels were identified by positive staining for vWF and morphology. The individual different fields for each tissue preparation, and microvessel density were expressed as the total microvessel count in the ten fields.

2.4. Determination of Cytokines Present in Mice Serum

Serum levels of tumor necrosis factor alpha (TNF-alpha) and interleukin-12 (IL-12) were measured 21 days after tumor cells transplantation. Under ether anesthesia mice were bled 4 h after the last G-CSF injection, and the levels of TNF-alpha and IL-12 in serum samples were determined using ELISA kits (Bio-Source International, Inc. Camarillo, CA). Each assay was performed according to the manufacture's instructions. The sensitivity of the TNF-alpha and IL-12 ELISA kits are 3.0 pg/ml and 2.0 pg/ml, respectively. All samples and standards were run in duplicate.

2.5. Statistical Analysis

The data was expressed as the mean \pm SD. P values were calculated using the Student's t-test, and $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. *In Vitro* Effect of G-CSF on Tumor Growth

In vitro effect of G-CSF on tumor cell proliferation and the absence of G-CSF receptor mRNA in lung cancer cells.

The direct effect of G-CSF on tumor cell proliferation was determined by MTT assay in two mouse cell lines. We found that LL-2 and KLN-205 cell proliferation was unchanged significantly in the presence of various concentrations of G-CSF (**Figure 1**). To ensure that the results were due to the absence of the G-CSF receptor, we investigated the G-CSF receptor mRNA in these two cell lines by RT-PCR. A 567 bp fragment of G-CSF receptor mRNA was detected only in unseparated bone marrow cells (positive control), and was not detected in these two cell lines (**Figure 2**).

3.2. *In Vivo* Effect of G-CSF on Tumor Growth

Next, to evaluate the effects of G-CSF on lung cancer *in vivo*, we studied the tumor growth in lung cancer-bearing mice model treated with G-CSF and also in

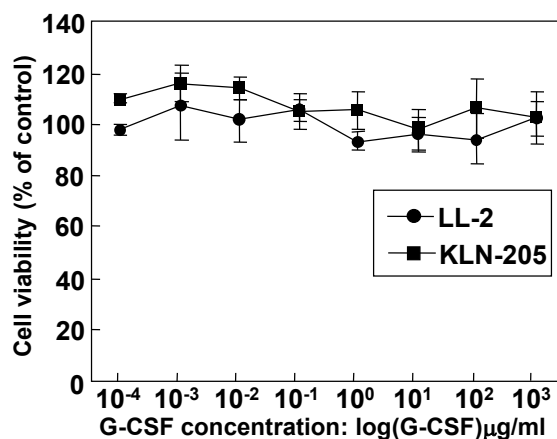


Figure 1. Examination of the in vitro lung cancer cell proliferation. The cultured cell is cultured with G-CSF of various kinds of density, and the cell proliferation is examined in MTT assay and is displayed in percent.

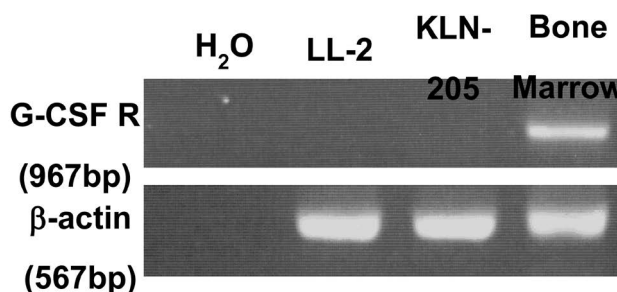


Figure 2. Detection of G-CSF receptor mRNA by RT-PCR. The presence of 567 bp fragment indicates G-CSF receptor mRNA. Positive control: unseparated murine bone marrow cell. Murine lung cancer cell lines LL-2 and KLN-205 could not detect G-CSF receptor mRNA. β-actin mRNA (967bp) was detected in all tested cell lines.

investigated the combination effects of G-CSF and cisplatin the latter of which is commonly used in chemotherapy of lung cancer. In a previous study, six of ten mice died at 10 mg/kg CDDP and no mice died at 5 mg/kg CDDP. Thus, the maximum tolerable dose (MTD) was regarded as being close to 5 mg/kg. Groups of mice were intraperitoneally given 5 mg/kg CDDP or saline per week starting 2 hours after tumor cell transplantation. Then, 2 hours after CDDP or saline injection the mice were intraperitoneally given 30 micro/kg G-CSF or saline per day. Tumor growth was markedly inhibited in the CDDP and CDDP+G-CSF treatment group compared with the saline control group. Concurrent administration of G-CSF significantly enhanced the tumor suppressing effect of CDDP in early stage tumor growth. 7 days after tumor cells transplantation, the tumor volume were 6.84 ± 9.07 for CDDP plus G-CSF treatment VS 16.34 ± 10.29 mm³ for CDDP alone ($p = 0.047$) (Figure 3).

3.3. Mechanism of the Effectiveness

To evaluate the effect of G-CSF on tumor-associated angiogenesis, immunostaining for vWF, an established endothelial cell marker, was performed. As

shown in **Figure 4**, in tumors from mice 7 days after G-CSF treatment, vessels were usually found in the actively proliferating peripheral region of the tumors, whereas the middle tumor parenchyma showed scant vessel distribution. In contrast, tumors from the control mice showed only few vessels even in peripheral tumor tissues (53.2 ± 5.3 versus 35.4 ± 3.7) $p < 0.05$.

Recent papers reported that G-CSF modulates the balance between Th1 and Th2 cells and down-regulates the Th1 driving cytokine [27] [28], so, we studied the representative Th1 cytokine, TNF-alpha and IL-12. The concentrations of TNF- α and IL-12 in the serum of tumor-bearing mice were estimated using ELISA. As shown in **Figure 5**, there was no statistically significant difference for

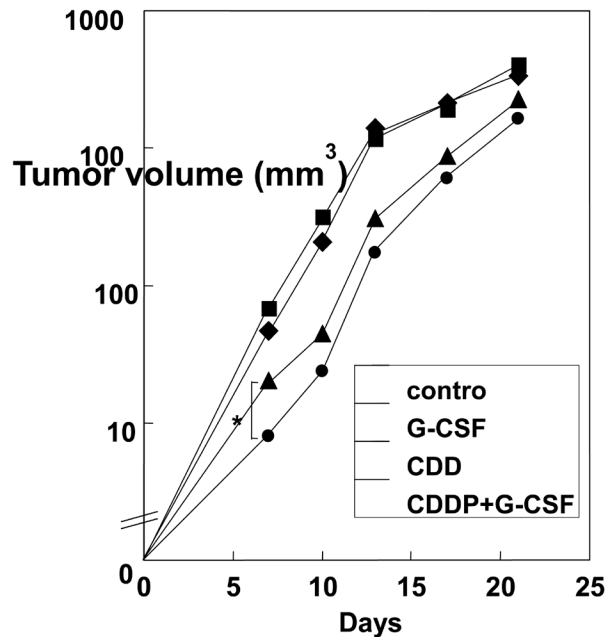


Figure 3. Effect of treatment with G-CSF and CDDP on growth of lewis lung (LL-2) carcinoma. Tumor size was measured by calipers on the longest superficial diameter (a) and smallest superficial diameter (b) Tumor volume was expressed as $a \times b^2 \times 0.52 \text{ mm}^3$. Filled diamond: saline, filled square: G-CSF treatment, filled triangle: CDDP treatment and filled circle: G+C (G-CSF and CDDP) treatment.

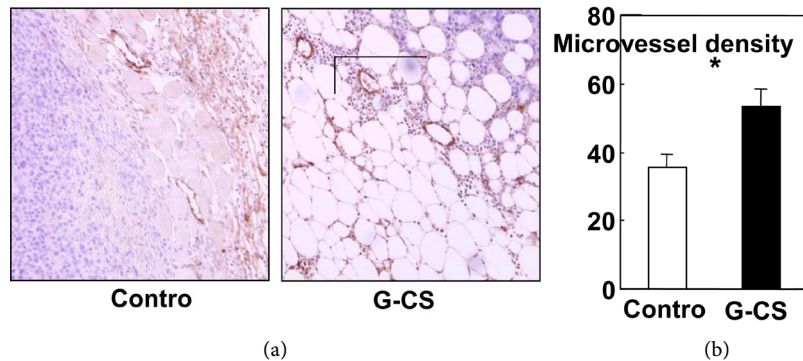


Figure 4. Effects of G-CSF on tumor angiogenesis. (a) The blood vessels were stained with vWF antibody ($\times 100$); (b) Microvessel density was assessed in the highest staining for vWF positive area in ten fields. * < 0.05 .

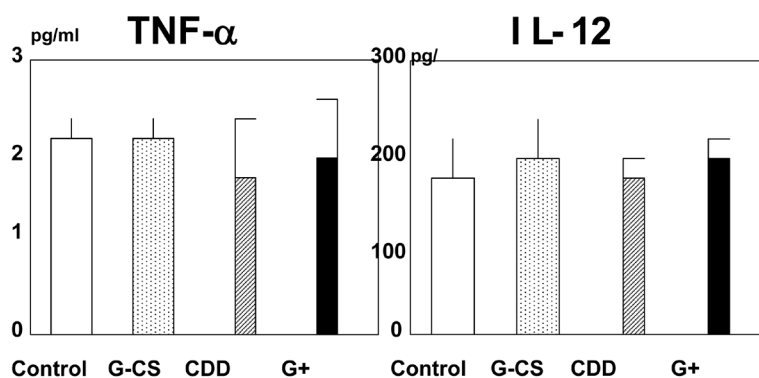


Figure 5. Serum concentration of TNF-alpha and IL-12. The serum concentration of TNF-alpha and IL-12 that were taken from 21 days after drugs treatment. White column.

the IL-12 and TNF- α concentrations in serum taken 21 days after drug treatment among the 4 groups ($p > 0.05$).

4. Discussion

4.1. Influence on Cancer Increase of the G-CSF

Generally, the hematopoietic growth factors are widely used for neutrophil recovery during chemotherapy in solid tumors, and there were few reports whether these growth factors affect solid tumor growth and they compromise anti-cancer drug effects. In the present study, the effects of G-CSF on lung cancer growth were investigated. We found that the murine non-small lung cancer cell lines LL-2 and KLN-205 were not affected by G-CSF treatment *in vitro*. Moreover, RT-PCR revealed the absence of G-CSF receptor mRNA, suggesting that G-CSF has no direct effect on the growth of these lung cancer cell lines due to the absence of the G-CSF receptor. For *in vivo* studies, G-CSF alone did not alter tumor growth, whereas concurrent administration of G-CSF with CDDP significantly enhanced the tumor suppressing effect of CDDP in early stage tumor growth. CDDP is cytotoxic agent and a key drug in the chemotherapy of lung cancer. It is generally accepted that cytotoxicity of CDDP is mediated through induction of apoptosis and arrest of cell cycle resulting from its interaction with DNA, such as the formation of CDDP-DNA adducts, which activates multiple signaling pathways, including those involving p53, Bcl-2 family, caspase, cyclins, MAPK and PI3K/Akt [29] [30] [31].

4.2. Mechanism of the Effectiveness

To determine the mechanisms responsible for the enhanced effect of tumor growth inhibition for concurrent administration of G-CSF with CDDP, we focused on the effect of G-CSF on tumor-associated angiogenesis as measured by vWF immunostaining, and found that treatment with G-CSF significantly enhanced tumor neovascularization. Bussolino *et al.* showed that G-CSF stimulates DNA synthesis followed by the endothelial cell proliferation [32], and Natori *et al.* showed that bone marrow-derived endothelial progenitor cells play a role in

enhanced tumor angiogenesis in an implanted colon cancer cell mouse model [33]. So, we also considered that G-CSF promotes the angiogenesis by stimulating DNA synthesis of endothelial cell and mobilizing bone marrow-derived endothelial progenitor cells. As the reason of the enhanced anticancer effect by addition of G-CSF, we thought that the enhancement of tumor neovascularization by addition of G-CSF increased the concentration and the exposure time of CDDP within tumor in early stage. And in late stage, the tumor neovascularization did not occur inside of tumor and the concentration of CDDP inside tumor was thought to be very low, so the significant difference of the tumor size was not seen between CDDP group and CDDP + G-CSF group.

It is recently reported that G-CSF has the function not only to mobilize the hematopoietic stem cells but also to immunomodulate the balance between T helper 1 (Th1) and T helper 2 (Th2) cells in favor of Th2 cells [27] [28] [34] [35]. In the present study, we did not detect the significant difference of TNF-alpha and IL-12 concentrations in serum taken 21 days after drug treatment among the 4 groups. As the reason of discrepancy between our data and past report, we thought the character of C57BL/6 mice, which has Th1 dominant immune system and concentration and duration of G-CSF treatment, but it is not clear for this reason of discrepancy and more research must be needed.

4.3. Limit of This Study

It is necessary for the limit of this study to examine the increase of cancer when I used other anticancer agents and anticancer agents in future in the examination of the increase of the cancer cell when the G-CSF used it together not taking effect and G-CFS together.

In summary, our results provide evidence that G-CSF as a growth factor does not promote tumor cell proliferation. Concurrent (Combination) administration of G-CSF significantly enhances the tumor suppressing effect of CDDP in early stage tumor growth. Thus, concurrent (combination) administration of G-CSF with anticancer agents is a safe and effective method for reducing chemotherapeutic agent-induced myelosuppression. Although further studies are required to determine whether this effect of G-CSF is a common feature against lung cancer and the solid tumors of the other organs, in this time, our study suggested a novel importance of G-CSF treatment against cancer therapy.

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