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Direct and indirect effects of recombinant human granulocyte-colony stimulating factor on in vitro colony formation of human bladder cancer cells

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Abstract. Although the present experimental use of recombinant human granulocyte-colony-stimulating factor (rG-CSF) has been proven to alleviate the myelosuppression induced by antitumor chemotherapy, it is also believed to stimulate growth of some nonhematopoietic tumor cells. We investigated both the direct and indirect effects of rG-CSF on in vitro colony formation of human bladder cancer cell lines using a modified human tumor clonogenic assay. Peripheral blood mononuclear cells (PBMC) were used as feeder cells (a mixture of 5×10^4 monocytes/dish and 5×10^5 lymphocytes/dish obtained from healthy donors). Human bladder cancer cell lines KK-47, TCCSUP and T24, all derived from human transitional-cell carcinomas, were incubated continuously with various concentrations of rG-CSF ranging from 0.01 ng/ml to 10 ng/ml both with and without PBMC for 7–21 days. The concentrations of rG-CSF used were chosen as being in the range of achievable serum concentrations in patients treated with rG-CSF. At the end of incubation, colonies were counted under an inverted phase-contrast microscope, and an increase in the number of colonies in comparison with the control was used to evaluate the effects of rG-CSF. Results were expressed as a percentage of controls. rG-CSF in the upper layer at concentrations ranging from 0.1 ng/ml to 10 ng/ml stimulated the colony formation of all the cancer cell lines tested in the absence of PBMC in the feeder layer, whereas cells with PBMC in the feeder layer were significantly stimulated more than those without PBMC in the feeder layer ($P < 0.05$) up to a certain concentration, which varied from cell line to cell line. At higher concentrations of rG-CSF, no further stimulation but, on the contrary, a decrease in colony formation was observed in cells with PBMC in the feeder layer in all the cell lines tested. Colony formation in KK-47 and T24 cell lines was significantly inhibited at 5 ng/ml and/or 10 ng/ml rG-CSF compared with cells without PBMC in the feeder layer. Our results suggest that rG-CSF may have both direct and indirect stimulatory effects on the growth of human bladder cancer cell lines in vitro. The results obtained also raise the possibility of adverse effects of rG-CSF in bladder cancer patients whose malignant cells may be directly and indirectly stimulated by this factor while it is being used clinically to alleviate the myelosuppression induced by antitumor chemotherapy.

Key words: rG-CSF – Human bladder cancer cells – In vitro – Proliferating activity

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Introduction

Granulocyte-colony-stimulating factor (G-CSF) is a glycoprotein [17] that specifically induces proliferation and differentiation of neutrophil colonies [7]. The DNAs complementary to the respective mRNAs have been cloned, and recombinant granulocyte-colony-stimulating factor (rG-CSF) is being used clinically to alleviate the myelosuppression induced by antitumor chemotherapy [5]. G-CSF is naturally produced by monocytes on stimulation with endotoxin [30], interleukin-3, granulocyte/macrophage-colony-stimulating factor (GM-CSF) [21] or interleukin-4 [31], by fibroblasts on stimulation with interleukin-1 [13] or tumor necrosis factor [33], and by endothelial cells [34]. Although the experimental use of rG-CSF has been proven to alleviate the myelosuppression induced by antitumor chemotherapy, it is also believed to stimulate the growth of some nonhematopoietic tumor cells [3]. Also, various tumors have been reported to produce colony-stimulating factors that might in turn stimulate their growth by paracrine or autocrine mechanisms [14, 32]. On the other hand, it was reported that some kinds of leukemic cells did not respond to G-CSF [2].

Treatment for advanced urinary bladder cancer has used various combinations of chemotherapy [28]. More recently, escalated-dose combination chemotherapy using rG-CSF has been reported [16, 24, 26]. The effect of rG-CSF on

human urinary bladder cancer cells is not yet clearly defined. It is therefore considered to be very important to evaluate the effects of rG-CSF on the growth of human urinary bladder cancer cells before any rational clinical trials using rG-CSF are undertaken to treat patients with advanced urinary bladder carcinoma. We therefore investigated both the direct and indirect effects of rG-CSF on the in vitro colony formation of human urinary bladder cancer cell lines using a modified human tumor clonogenic assay.

Materials and methods

Cell lines and their maintenance. Three kinds of human bladder cancer cell line were used in our study. Cell lines KK-47, TCCSUP and T24 are all derived from human transitional-cell carcinomas of the urinary bladder and represent histopathological grades G1, G4 and G3 respectively. They have been detailed elsewhere [29, 4, 18]. All were *Mycoplasma*-free. In our laboratory, tumor cell lines are routinely screened for *Mycoplasma* contamination using a *Mycoplasma* stain kit (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan). The method has been detailed by Chen [6]. In addition, as a preventive measure we cultivate cell lines with *Mycoplasma* removal agent (M.R.A., Dainippon) for 1 week before starting any experiments using cell lines [1, 22]. All the tumor cell lines were maintained as a monolayer culture in a 75-cm^2 polystyrene tissue-culture flask (Corning 25110, Corning, N.Y.) suspended in RPMI-1640 culture medium (Flow Laboratories, McLean, Va.) supplemented with 10% heat-inactivated fetal calf serum (lot no. 0130103, Flow Laboratories), penicillin (100 units/ml) and streptomycin (100 µg/ml) (Gibco, Grand Island, N.Y.) at 37° C in a humidified atmosphere of 5% CO_2. Tumor cells from sub-confluent flasks were used throughout the experiments. The monolayer culture was dispersed by trypsinization using trypsin/ethylenediaminetetraacetic acid (EDTA) solution: 0.5 g/l trypsin and 0.2 g/l NaEDTA dissolved in magnesium and calcium-free phosphate-buffered saline (PBS) (DPBS, Whittaker, Walerville). The plating density of each cell line was between 5×10^2 and 5×10^4 cells/dish.

Biological reagent. Purified human recombinant G-CSF, expressed in Chinese hamster ovary cells [17], was kindly provided by Chugai Pharmaceutical Co. (Tokyo, Japan). It was diluted in RPMI-1640 medium (Flow Lab.) in such a way that the final concentrations of rG-CSF ranged from 0.01 ng/ml to 10 ng/ml. Specific activity was 1.49×10^8 units/mg. The concentrations of rG-CSF used were chosen as being in the range of achievable serum concentrations in patients treated with rG-CSF [27].

Preparation of feeder cells. A 30-ml sample of heparinized human peripheral blood was obtained each time from healthy donors. Blood samples were layered on 30 ml lymphocyte separation medium (LSM, Litton Bionetics, Kensington, Md., USA) and centrifuged at 500 *g* for 30 min at room temperature. The mononuclear cell layer was collected by pipette. After washing with PBS without calcium and magnesium, cells were resuspended and decanted into a 75-cm^2 polystyrene tissue-culture flask. Cells were then incubated and allowed to adhere at 37° C in a humidified atmosphere of 5% CO_2 for 1 h. After removal of nonadherent cells, the adherent monolayer was rinsed three times with warm PBS. The adherent cells were removed from the flask by a cell scraper (Falcon 3086, Becton Dickinson) and plated in the feeder layer at a density of 5×10^4 cells/dish. More than 90% of this cell population was monocytes as determined by morphological examination using Giemsa-stained preparations. Nonadherent cells were also plated in the feeder layer at a density of 5×10^5 cells/dish.

Human tumor clonogenic assay. The method originally described by Hamburger and Salmon [9] was used in this study with slight modification. Briefly, the feeder layer was prepared in 35-mm petri dishes (Falcon 3801, Becton Dickinson, Sunnyvale, Calif.) using RPMI-1640 culture medium (Flow Lab.) supplemented with 25% heat-inactivated fetal calf serum (Flow Lab.) and penicillin (100 units/ml), streptomycin (100 µg/ml) (Gibco) in 0.6% agar (Difco Laboratories, Detroit). Tumor cells were suspended in the upper layer with various concentrations of rG-CSF in 0.3% agar (Difco) prepared with the same medium as the feeder layer. Agar double-layer cultures of tumor cells were incubated at 37° C in a humidified atmosphere of 5% CO_2 for 7–21 days. Each experiment was conducted in triplicate and six plates were prepared as controls. The presence of a single-cell suspension was almost ensured at the time of plating by visual inspection using inverted microscopy. Also, in order to distinguish non-colony cell aggregates, three plates were fixed with 10% formaldehyde before cultivation as positive controls. At the end of incubation, colonies (a colony is defined as aggregates of 40 or more cells) were counted under an inverted phase-contrast microscope (Nikon, Tokyo). The true number of colonies was calculated as the number of aggregates on the positive control subtracted from the number of colonies on the experimental plate. Results were expressed as a percentage of the control value. Occasionally, at least two independent investigators evaluated colony morphology and number. The entire procedure was repeated three times for each cell line as a means of reproducibly measuring the effects. In order to distinguish the direct and indirect effects of rG-CSF, a direct effect was defined as the percentage of colony growth in the absence of feeder cells, while a combined direct and indirect effect was defined as the percentage of colony growth in the presence of feeder cells.

Statistical analysis. All data were expressed as the mean ± the standard deviation of three separate experiments performed in triplicate each time. The data obtained were analyzed for statistical differences by using a two-sample Wilcoxon test. A P value of less than 0.05 was regarded as statistically significant.

Table 1. Effect of recombinant human granulocyte-colony-stimulating factor (rG-CSF) on colony formation of KK-47, TCCSUP and T24 in soft agar in the absence of peripheral blood mononuclear cells (PBMC) in the feeder layer

Concentrations of rG-CSF (ng/ml)	Colonies (% of controls ± SD)		
	KK-47	TCCSUP	T24
0.01	104.23±1.89	116.04±19.46	102.18±16.70
0.05	105.01±3.56	117.97±10.94*	120.03±14.52*
0.1	110.41±5.04*	116.20±11.83*	131.56±16.27*
0.5	113.62±3.58*	115.13±17.45*	137.17±17.07*
1.0	117.25±2.20*	125.58±17.21*	140.88±15.40*
5.0	121.94±2.86*	120.42±13.46*	142.20±13.38*
10	125.96±3.26*	117.98±15.84*	142.26±16.00*

The values are given as percentages of controls ± SD of three separate experiments performed in triplicate each time
* $P<0.05$, two-sample Wilcoxon test when compared with the controls

Results

Our experiment was designed to assess both the direct and combined direct and indirect effects of rG-CSF on the colony-forming ability of human bladder cancer cells in vitro. The median colony-forming efficiency, as a percentage of the control, was 63.6% (range 58%–68%), 5.8% (4.4%–6.1%) and 0.38% (0.32%–0.42%) respectively for KK-47, TCCSUP and T24 cell lines. Cell clumps were very few and it ranged from 1–3 with a median of 2/dish.

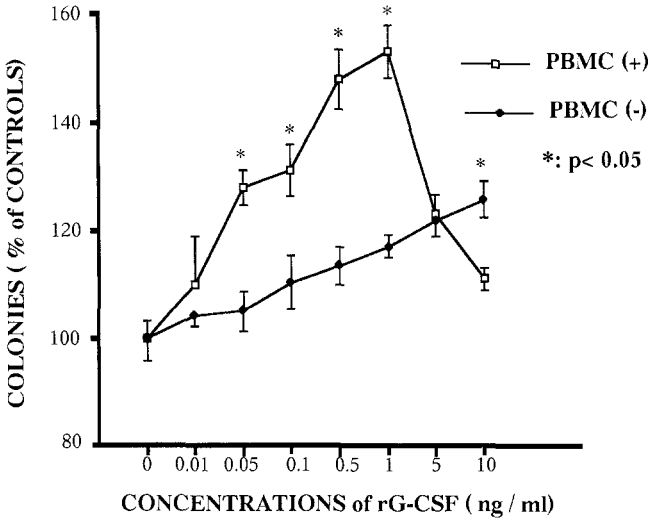

Fig. 1. Effect of recombinant human granulocyte-colony-stimulating factor (*rG-CSF*) on colony formation of KK-47 in both the presence and absence of peripheral blood mononuclear cells (*PBMC*) in the feeder layer. The values are given as percentages of controls ± SD of three separate experiments performed in triplicate each time. *PBMC*, a mixture of 5×10^4 monocytes/dish and 5×10^5 lymphocytes/dish obtained from healthy donors. * $P<0.05$, two-sample Wilcoxon test when compared with cells grown without PBMC

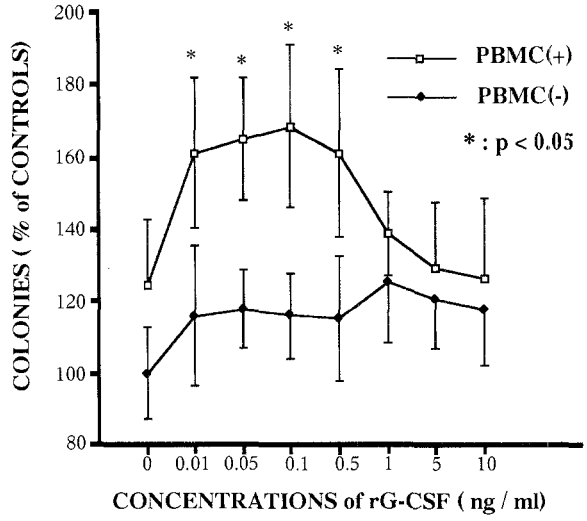

Fig. 2. Effect of rG-CSF on colony formation of TCCSUP in both the presence and absence of PBMC in the feeder layer. The values are given as percentages of controls ± SD of three separate experiments performed in triplicate each time. *PBMC*, a mixture of 5×10^4 monocytes/dish and 5×10^5 lymphocytes/dish obtained from healthy donors. * $P<0.05$, two-sample Wilcoxon test when compared with cells grown without PBMC

Direct effects of rG-CSF

KK-47, TCCSUP and T24 were incubated with various concentrations of rG-CSF in the absence of PBMC in the feeder layer. rG-CSF stimulated the colony formation of all the cancer cells. The increase in the percentage of colonies was different for various cell lines at the same concentrations of rG-CSF (Table 1). A statistically significant difference in colony formation was observed between cells with rG-CSF at concentrations ranging from 0.1 ng/ml to 10 ng/ml and the control in the KK-47 cell line ($P < 0.05$). The same significant differences in colony formation were observed in TCCSUP and T24 cell lines with rG-CSF at concentrations ranging from 0.01 ng/ml to 10 ng/ml and 0.05 ng/ml to 10 ng/ml respectively.

Table 2. Effect of rG-CSF on colony formation of KK-47, TCCSUP and T24 in soft agar in the presence of PBMC in the feeder layer

Concentrations of rG-CSF (ng/ml)	Colonies (% of controls ± SD)		
	KK-47	TCCSUP	T24
0.00	100.00±3.14	124.55±18.18	99.44±12.27
0.01	110.00±9.08	161.16±20.83*	131.00±15.23*
0.05	128.02±3.25*	165.27±17.03*	139.16±25.90*
0.1	131.32±4.85*	168.60±22.51*	166.56±28.35*
0.5	148.02±5.60*	161.28±23.35*	156.97±17.15*
1.0	153.30±4.78*	138.95±11.64*	143.13±23.82*
5.0	123.09±3.61	129.47±18.40	103.87±24.12
10	111.15±2.21	126.55±22.25	87.85±11.09

The values are given as percentages of controls ± SD of three separate experiments performed in triplicate each time
* $P<0.05$, two-sample Wilcoxon test when compared with the controls

Effects of only PBMC in the feeder layer

When KK-47 and T24 cells were incubated with feeder cells only, no increase in the number of colonies was observed when compared with that of control grown without PBMC in the feeder layer (Table 2). Although an increase in the percentage of colonies was observed in the case of TCCSUP cells, it was not statistically significant (Table 2).

Combined direct and indirect effects of rG-CSF

To assess the combined direct and indirect effects of rG-CSF, cells were incubated with various concentrations of this drug in the presence of PBMC consisting of monocytes and lymphocytes in the feeder layer. Compared with the control, the growth of colonies in all cell lines tested was significantly stimulated by rG-CSF up to a certain concentration, which varied from cell line to cell line (Table 2, $P < 0.05$). The maximum stimulatory effect was different for various cell lines. A statistically significant increase in colony formation was observed between cells with PBMC and rG-CSF at concentrations ranging from 0.05 ng/ml to 1 ng/ml and cells without PBMC in the KK-47 cell line (Fig. 1, $P < 0.05$). The same significant increases in colony formation were observed at concentrations of rG-CSF ranging from 0.01 ng/ml to 0.5 ng/ml in the TCCSUP cell line and from 0.01 ng/ml to 0.1 ng/ml in the T24 cell line (Figs. 2 and 3). No further stimulation but, on the contrary, a decrease in colony formation was observed in KK-47, TCCSUP and T24 cell lines at concentrations of more than 1, 0.1 and 0.1 ng/ml respectively (Figs. 1–3). Colony formation in the KK-47 and T24 cell lines was significantly inhibited at 5 ng/ml and/or 10 ng/ml compared with cells without PBMC in the feeder layer (Figs. 1 and 3).

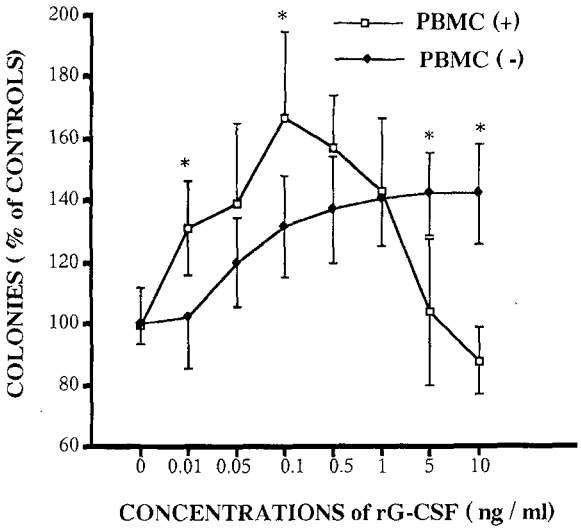

Fig. 3. Effect of rG-CSF on colony formation of T24 in both the presence and absence of PBMC in the feeder layer. The values are given as percentages of controls ±SD of three separate experiments performed in triplicate each time. *PBMC*, a mixture of 5×10^4 monocytes/dish and 5×10^5 lymphocytes/dish obtained from healthy donors. * $P<0.05$, two-sample Wilcoxon test when compared with cells grown without PBMC

There was no evidence that colonies in stimulated specimens comprised hematopoietic cells. Control experiments with PBMC in the feeder layer and rG-CSF in the upper layer without tumor cells did not reveal any colony formation.

Discussion

In clinical practice rG-CSF is a very useful drug to combat the myelosuppression induced by antitumor chemotherapy. This drug is also believed to stimulate growth of some nonhematopoietic cells. Urinary bladder cancer cells are reported to express functional receptors for G-CSF [20]. GM-CSF, a hematopoietic growth factor, had significant stimulatory effects on the proliferation of bladder cancer cells in vitro [11]. G-CSF also stimulated the colony formation of primary human tumor in vitro [12]. In the present study rG-CSF stimulated the colony formation of all the cancer cells tested both in the presence and absence of PBMC. And cells with PBMC in the feeder layer were stimulated more than those without PBMC. It has been reported that G-CSF acts by binding to the receptors present on the surface of G-CSF-sensitive cells [19]. An indirect mechanism of enhancement of tumor growth in vivo by G-CSF has also been reported [25]. Our study demonstrates that rG-CSF may have both direct and indirect stimulatory effects on colony formation. The direct effect might have been mediated through binding to the receptors present on the surface of cells. With our present experimental design, however, it is difficult to explain the mechanism of the indirect effect. It was reported by Hamburger et al. [10] that a soluble factor was released from monocytes and this factor in turn stimulated colony formation of SW-13 cells in vitro. Stimulation of tumor cell growth in humans by a factor derived from monocytes was observed by Sandru et al. [23]. Monocytes require an additional signal to stimulate release of growth-promoting factor(s) [8]. We hypothesize from our results that some diffusible factor(s) were released in the soft agar from PBMC upon stimulation by rG-CSF and this in turn might have caused the stimulation of colony formation, because PBMC alone in the feeder layer did not stimulate colony formation in any of the cancer cells tested. Our data suggest that rG-CSF may be a potential mediator of release of some diffusible factor(s) from PBMC. rG-CSF stimulated colony formation in cells with PBMC in the feeder layer up to a certain concentration, which varied from cell line to cell line. Stimulation of colony formation of cells with PBMC was not observed with higher concentrations of rG-CSF. On the contrary, colony formation of all cell lines decreased gradually at concentrations of more than 1, 0.1 and 0.1 ng/ml rG-CSF, respectively, for the KK-47, TCCSUP and T24 cell lines. We speculate that this decrease might have been due to exhaustion of available factor(s) released from PBMC or to the presence of some cytotoxic factor(s). There was some heterogeneity in the sensitivity of the cell lines to rG-CSF. This heterogeneous response may be due to different receptor numbers and affinities and, also, to different responsiveness to the diffusible factor(s) released from PBMC.

The management of invasive and metastatic urinary bladder cancer patients remains one of the most controversial and challenging problems in urological practice. The regimen of methotrexate, vinblastine, doxorubicin and cisplatin (M-VAC) [28], first piloted in the early 1980s, ushered in new hope for patients with metastatic urinary bladder carcinoma. With the passage of time and experience it has become clear that M-VAC has not fulfilled its early promise [15], so investigators have began to look for more effective regimens to treat patients with advanced disease. More recently, escalated-dose combination chemotherapy using rG-CSF to combat myelosuppression has been reported but with limited success [16, 24, 26]. There might be many reasons for this limited success. We would like to suggest from our study that the adverse effects of rG-CSF might be one of the reasons in this context. On the other hand, some investigators have postulated that, since G-CSF stimulated DNA synthesis of cancer cells, administration of G-CSF prior to treatment using an S-phase-specific cytotoxic agent may render neoplastic cells more sensitive to therapy [20, 3]. It is also possible that the use of G-CSF during escalated-dose chemotherapy may make G-CSF-sensitive cells more susceptible to the chemotherapeutic agent. However, the problems that may arise in these patients as a result of tumor heterogeneity in response to rG-CSF cannot be fully ruled out. Before any final conclusion is drawn and any rational clinical trials using rG-CSF are conducted to treat patients with advanced urinary bladder carcinoma, more studies evaluating the effects of rG-CSF both in vitro and vivo are warranted. Our results might provide a valuable reference in light of the initiation of widespread clinical trials including escalated-dose combination chemotherapy using rG-CSF in urinary bladder cancer patients. The present study also supports the notion that rG-CSF can stimulate the clonal growth of some nonhematopoietic cells in vitro.

We conclude from our study that rG-CSF may have both direct and indirect stimulatory effects on the growth of human bladder cancer cells in vitro. rG-CSF may stimulate the growth of tumor cells remaining after chemotherapy while it is being used clinically to alleviate the myelosuppression induced by antitumor chemotherapy in bladder cancer patients.

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