Autocrine Growth of Transitional Cell Carcinoma of the Bladder Induced by Granulocyte-Colony Stimulating Factor

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ABSTRACT

Granulocyte-colony stimulating factor (G-CSF) produced by nonhematopoietic malignant cells has been reported to be capable of inducing a leukemoid reaction in the host through intense stimulation of leukocyte production. Furthermore, this is frequently associated with aggressive tumor cell growth and a detrimental clinical outcome. In this study, we identified bladder cancer cells producing G-CSF with the expression of the functional receptor, which provides direct evidence of autocrine growth of bladder cancer cells induced by G-CSF. The cancer cells used in this study were obtained from a 76-year-old man who had a metastatic transitional cell carcinoma of the bladder and who demonstrated marked leukocytosis; his peripheral blood leukocyte count was 94,900 leukocytes/mm³, and his serum G-CSF level was 103 pg/ml. Immunohistochemical study of the cancer tissue obtained by biopsy with the use of a mAb against recombinant human G-CSF was performed to identify the exact cell type responsible for G-CSF production. Sections of the 5% formalin-fixed and paraffin-embedded bladder tumor tissue specimen were studied by using the avidin-biotin-peroxidase method. Mouse anti-human G-CSF mAb (KW341) provided by Kyowa Hakko Co, Ltd. (Tokyo, Japan) was used as the primary antibody at a dilution of 1:50. To confirm the specificity of the immunohistochemical study, tumor specimens from SCID mice transplanted with CHO cells transfected with human G-CSF cDNA (17) were examined as a positive control, and as a negative control, mouse IgG was used as the primary antibody instead of anti-G-CSF antibody.

MATERIALS AND METHODS

The cancer cells used in this study were obtained from a 76-year-old man with metastatic transitional cell carcinoma of the bladder who demonstrated marked leukocytosis; his peripheral blood leukocyte count was 94,900 leukocytes/mm³, and his serum G-CSF level was 103 pg/ml. Immunohistochemical study of the cancer tissue obtained by biopsy with the use of a mAb against recombinant human G-CSF was performed to identify the exact cell type responsible for G-CSF production. Sections of the 5% formalin-fixed and paraffin-embedded bladder tumor tissue specimen were studied by using the avidin-biotin-peroxidase method. Mouse anti-human G-CSF mAb (KW341) provided by Kyowa Hakko Co, Ltd. (Tokyo, Japan) was used as the primary antibody at a dilution of 1:50. To confirm the specificity of the immunohistochemical study, tumor specimens from SCID mice transplanted with CHO cells transfected with human G-CSF cDNA (17) were examined as a positive control, and as a negative control, mouse IgG was used as the primary antibody instead of anti-G-CSF antibody.

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The abbreviations used are: G-CSF, granulocyte-colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; RT-PCR, reverse transcription-PCR; BrdUrd, bromodeoxyuridine; LI, labeling index; SCID, severe combined immunodeficiency; CHO, Chinese hamster ovary.

INTRODUCTION

G-CSF³ produced by nonhematopoietic malignant cells has been reported to be capable of inducing a leukemoid reaction in the host through intense stimulation of leukocyte production (1–11). This is frequently associated with aggressive tumor cell growth and a detrimental clinical outcome (8–12).

Varieties of nonhematopoietic malignant tumors, including bladder carcinoma (2–4), hepatoma (5), mesothelioma (6), squamous cell carcinoma of the oropharynx (7), melanoma (8), glioblastoma (9), and sarcoma (10, 11), have been demonstrated to secrete G-CSF in amounts large enough to cause a significant systemic hematopoietic effect. In addition, receptors for G-CSF have also been confirmed on the cell surfaces of several nonhematopoietic cell types, including human placenta and trophoblastic cells (13), human vascular endothelial cells (14), and cell lines derived from human small cell carcinoma of the lung (15). Previously, we reported the expression of functional G-CSF receptors in transitional cell carcinoma of the bladder (16).

The above observations lead naturally to the tempting speculation that simultaneous acquisition of the ligand (G-CSF) production and its receptor expression by a malignant tumor may provide a strong autocrine growth advantage. This study addresses our recent observations, which strongly suggest that such autocrine growth promotion of malignant tumor cells by G-CSF does, in fact, take place.
Stimulation of cultured cancer cells by exogenous G-CSF administration and neutralization of its growth-promoting activity by anti-G-CSF antibody under serum-free conditions were studied. The proliferating activity of the cultured cancer cells was measured by the flow cytometric BrdUrd incorporation technique. The cells were incubated in 1 ml of a 1:1 mixture of RPMI 1640 and Eagle’s MEM without serum supplementation in 12-well culture dishes (well diameter, 22 mm; Corning) at 37°C in a humidified atmosphere of 5% CO2 with 95% air. Serial concentrations of recombinant mutant human G-CSF, kindly provided by Kyowa Hakko Kogyo Co., Ltd. (KW-2228; 3, 7), were added every 24 h for a total of three times. Twenty-four h after the final G-CSF treatment, BrdUrd was added to each culture well at a final concentration of 5 μg/ml, and incubation was continued for another hour. The cells were harvested with 0.25% trypsin and 1 mM EDTA and were then washed twice. The cells were subsequently stained with FITC-labeled anti-BrdUrd antibody and then poststained with 0.5% propidium iodide. The double-stained cells were analyzed with an Epics ELITE flow cytometer (Coulter, Hialeah, FL), and the LI, i.e., the number of cells stained with BrdUrd divided by the total estimated cell count, was calculated.

For the neutralizing test, 0.5 μg/ml concentrations of G-CSF were preincubated with or without serial concentrations of anti-human G-CSF antibody (IgG class; R&D Systems, Minneapolis, MN) before their addition to the cultured cells. The experiment was otherwise carried out in exactly the same way as the stimulation test. It was also demonstrated whether the presence of a specific anti-human G-CSF antibody would inhibit tumor cell proliferation. The antibody was added every 24 h for a total of three times to cell cultures with or without serial concentrations of anti-human G-CSF antibody (R&D Systems) under a serum-free condition. The experiment otherwise was carried out in exactly the same way as the neutralizing test. KU-7 cells (19) derived from human bladder cancer and not exhibiting functional G-CSF receptors were used as control cells.

RESULTS

The cancer cells used in this study were obtained from a 76-year-old man with metastatic transitional cell carcinoma of the bladder. The patient underwent radical cystectomy for invasive carcinoma of the bladder on June 28, 1993. Pathological analysis of the excised bladder...
G-CSF and G-CSF receptor mRNA expression on the cultured cancer cells were studied with the use of RT signals for both G-CSF and G-CSF receptor and were detected, as shown in Fig. 3, B and C. The RT-PCR product exhibited a specific G-CSF transcription signal of 278 bp and a G-CSF receptor signal of 727 bp in samples from the cultured cells. The RT-PCR product demonstrated a specific GM-CSF transcription signal of 441 bp (Fig. 3D) but no definitive GM-CSF receptor transcription signal of 621 bp (E).

Stimulation of cultured cancer cells by exogenous G-CSF administration and neutralization of its growth-promoting activity by anti-G-CSF antibody were studied by the flow cytometric BrdUrd incorporation technique. As shown in Fig. 4A, the BrdUrd LIs were 12.7, 14.0, and 16.7% for 0, 0.1, and 0.5 μg/ml G-CSF concentrations, respectively. Therefore, proliferation of the cultured cancer cells was stimulated by G-CSF. Meanwhile, when 0.5 μg/ml G-CSF was preincubated with serial concentrations of anti-G-CSF antibody, BrdUrd LIs were 17.2, 14.3, and 12.7% for 0, 10, and 50 μg/ml concentrations of anti-G-CSF antibody, respectively (Fig. 4B). Therefore, stimulation of cell proliferation by G-CSF was inhibited by anti-G-CSF antibody. The addition of 50 μg of anti-G-CSF antibody neutralized the cell growth stimulated by 0.5 μg/ml of G-CSF by 26.2%. Furthermore, when the cells were cultured with anti-G-CSF antibody, BrdUrd LIs were 11.7, 10.4, 9.5, and 8.9% at anti-G-CSF antibody concentrations of 0, 10, 50 μg, and 200 μg/ml, respectively (Fig. 5).

In addition, [3H]thymidine uptake of the cells was illustrated in Table 1. The uptake at 24-h incubation after the final G-CSF administration with 0.1 and 0.5 μg/ml was 5544.7 ± 680.1 and 6030.8 ± 409.5 cpm, respectively. These were significantly higher than those of controls (4760.6 ± 309.1 cpm; P < 0.05). In addition, 0.5 μg/ml G-CSF was preincubated with serial concentrations of anti-G-CSF antibody, and [3H]thymidine incorporations were 6180.8 ± 285.3, 4849.8 ± 216.5, and 4373.0 ± 278.1 cpm in 0, 10, and 50 μg/ml concentrations of anti-G-CSF antibody, respectively. [3H]thymidine incorporations with anti-G-CSF antibody cultures were

![Image](attachment:image.png)

**Fig. 3.** Detection of G-CSF mRNA and G-CSF receptor mRNA by the RT-PCR method. A, the β-actin-specific fragment was detected by PCR (20 cycles at 94°C for 1 min, 65°C for 1 min, and 72°C for 3 min) with primers 5'-GATATCGCCGGCTGCTGCGAC-3’ (forward primer) and 5’-CAGGAAGGAAAGGTGGAGATG-3’ (reverse primer); B, the G-CSF-specific 278-bp fragment was detected by PCR (40 cycles at 94°C for 1 min, and 50°C for 1 min) with 5’-CTGTGTCACCTCAAAG-3’ (forward primer) and 5’-GCCATCCTCGTTCTCC-3’ (reverse primer); C, the G-CSF receptor α-chain 727-bp fragment was detected by PCR (35 cycles at 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min) with 5’-ACAGTTCTCACCCTGATACCT-3’ (forward primer) and 5’-TGCTCTTCAAAGGCGTTAGACTA-3’ (reverse primer); D, the 441-bp GM-CSF-specific fragment was detected by the PCR (43 cycles at 94°C for 30 s, and 63°C for 1 min) with primers 5’-CTGGAGACTTCTGGTGGAGACCC-3’ (forward primer) and 5’-TGCTGGAAAGCCCATCAGGAGTC-3’ (reverse primer); and E, the 621-bp GM-CSF receptor α-chain fragment was detected by PCR (40 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min) with primers 5’-TGACACCTAACATCGTTCTCC-3’ (forward) and 5’-ACACCTGACTTACCTCCAATCA-3’ (reverse primer). To further confirm that the amplified products originated from the respective cDNA, they were subjected to appropriate restriction enzyme digestions. Size markers from top: 4, 3, 1.8, 1.1, 0.68, 0.38, 0.25, and 0.12 kb. RT-PCR exhibited a 278-bp band signal for G-CSF (B), a 727-bp band signal for the G-CSF receptor (C), and a 441-bp band of GM-CSF (D) in samples from the cultured cells. However, the 621-bp band of GM-CSF receptor was not identified in the sample from the cultured cells (E).
Fig. 4. Study of growth stimulation by exogenous G-CSF administration and neutralization of growth-promoting activity by anti-G-CSF antibody. The proliferative activity of the cultured cancer cells was measured by the flow cytometric BrdUrd incorporation technique. The double-stained cells were analyzed with the use of an Epics ELITE flow cytometer, and the LI, i.e., the number of cells stained by BrdUrd divided by the total estimated cell count, was calculated. The LIs of the cells treated with 0.5, 0.1, and 0 μg/ml (control) of G-CSF were 16.7, 14.0, and 12.7%, respectively (A). For the neutralizing test, 0.5 μg/ml concentrations of G-CSF were preincubated with or without serial concentrations of anti-human G-CSF antibody before their addition to the cultured cells. The experiment was otherwise carried out in exactly the same way as the stimulation test. As shown in B, the addition of 50 μg of anti-G-CSF antibody neutralized the cell growth stimulated by 0.5 μg/ml of G-CSF, reducing growth by 26.2%.

Furthermore, when anti-G-CSF antibody was added in the cultures every 24 h for three times, [³H]thymidine incorporations were 3750.8 ± 178.8 cpm for 10 μg/ml, 3326.0 ± 246.2 cpm for 50 μg/ml, and 3166.7 ± 113.0 cpm for 200 μg/ml anti-G-CSF antibody concentrations. These were significantly lower than those without anti-G-CSF antibody administration (4132.2 ± 231.4 cpm/well; P < 0.01). However, KU-7 cells did not demonstrate any inhibition of BrdUrd labeling or [³H]thymidine incorporation when anti-G-CSF antibody was cocultured.

Binding studies with the use of the radiolabeled recombinant G-CSF demonstrated the presence of a high-affinity G-CSF binding receptor on the cultured cancer cells (Fig. 6). Nonspecific binding (binding of 125I-labeled KW-2228 to the cells in the presence of G-CSF at 1000 ng/0.5 ml), which ranged between 6 and 18%, was subtracted from the total binding to determine the specific binding. The specific binding of the labeled KW-2228 was expressed as the

Fig. 5. Flow cytometric BrdUrd incorporation study on the effect of anti-G-CSF antibody on cell growth proliferation. Anti-G-CSF antibody was added in the cultures every 24 h for three times under a serum-free condition. In vitro BrdUrd labeling was performed 24 h after the final administration of anti-G-CSF antibody. The LI was estimated as described previously. BrdUrd LIs were 11.7, 10.4, 9.5, and 8.9% in anti-G-CSF antibody concentrations of 0, 10, 50, and 200 μg/ml, respectively.

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percentage of binding measured in the absence of unlabeled KW-2228 (Fig. 6A). The Scatchard plot analysis of the specific binding of \(^{125}\)I-labeled KW-2228 to the cells shown in Fig. 6B indicates that the cells harbor a single type of G-CSF receptor. The \(B_{\text{max}}\) calculated from the intercept of the slope with the abscissa on the Scatchard plot was 458 molecules/cell, and the \(K_d\) was 103 pm.

**DISCUSSION**

Varieties of nonhematopoietic malignant tumors have been demonstrated to secrete G-CSF (2-11). In addition, it has been reported that receptors for G-CSF have been confirmed on the cell surfaces of several nonhematopoietic cell types (13-15). Bladder cancer cells have been shown to secrete a variety of biological factors with no direct relation to urothelial cell origin including G-CSF (2-4), GM-CSF (2, 22), and various cytokines (22).

Previously, we have reported the expression of functional receptors for G-CSF in transitional cell carcinoma of the bladder (16). In our previous report, G-CSF receptors were expressed on two bladder cancer cell lines, and administration of G-CSF provided increased cell proliferation, as estimated by the \(^{[3]}\)H]thymidine incorporation method.

These previous observations lead naturally to the tempting speculation that the simultaneous acquisition of G-CSF production and expression of its receptor, by a malignant tumor, may enhance autocrine growth. However, Sato et al. (4) reported on G-CSF-producing bladder cancer, although they indicated that their study failed to demonstrate a crucial role for G-CSF in mediating a growth advantage for the tumor. Furthermore, Thacker et al. (23) demonstrated that the human osteosarcoma cell line MG63 responds to both G-CSF and GM-CSF in vitro. They indicated that retrovirally infected G-CSF-producing or GM-CSF-producing MG63 cells exhibited autostimulatory growing features, as measured by \(^{[3]}\)H]thymidine incorporation.

Stimulation of cultured cancer cells by exogenous G-CSF administration and neutralization of this growth-promoting activity by anti-G-CSF antibody, as demonstrated by both the flow cytometric BrdUrd incorporation technique and \(^{[3]}\)H]thymidine incorporation assay indicate that the proliferation of cultured cancer cells was stimulated by G-CSF, and that this stimulation was inhibited by anti-G-CSF antibody. In addition, binding studies performed with the use of radiolabeled recombinant G-CSF demonstrated the presence of a high-affinity G-CSF binding receptor on the cultured cancer cells.

These results strongly suggest that G-CSF production by the bladder cancer cells produced an autocrine growth advantage. The leukemoid reaction is a well-known paraneoplastic syndrome that has been demonstrated to be initiated by G-CSF production by cancer cells (1). Furthermore, the leukemoid reaction has been widely observed clinically to appear at an advanced stage of cancer in association with aggressive cell growth (4, 12). It is, therefore, deemed likely that the G-CSF production and G-CSF receptor expression exhibited by cancer cells play crucial roles in mediating the malignant progression of the nonhematopoietic cancer cells.

The histogenesis of transitional cell carcinoma of the bladder remains uncertain, although several theories have been proposed. Some authors have suggested that a metaplastic phenomenon presenting various degrees of differentiation may explain the malignant transition of transitional cell carcinoma (24). In addition, this concept is supported further by the tremendous potential of the transitional epithelium and transitional cell carcinoma to differentiate along several lines (25). The frequent presence of both squamous and glandular differentiation has long been recognized in transitional cell carcinoma. Furthermore, the presence of neuroendocrine (small cell) differentiation has also been reported (26). Hematopoietic differentiation of transitional cell carcinoma, resulting in acquisition of G-CSF production and G-CSF receptor expression, is another possibility supported by our observations.

| Table 1 Effects of G-CSF and anti-G-CSF antibody on \(^{[3]}\)H]thymidine incorporation by bladder cancer cells* |
|-----------------|-----------------|------------------|
| **G-CSF administration** | \(^{[3]}\)H]thymidine incorporation (cpm/well) | |
| Control | 4760.6 ± 309.1 |
| 0.1 µg/ml G-CSF | 5544.7 ± 680.1 |
| 0.5 µg/ml G-CSF | 6030.8 ± 409.5 |
| **G-CSF neutralization** | \(^{[3]}\)H]thymidine incorporation (cpm/well) | |
| Control (G-CSF 0.5 µg/ml alone) | 6180.8 ± 285.3 |
| 0.5 µg/ml G-CSF + 10 µg/ml anti-G-CSF Ab | 4849.8 ± 216.5 |
| 0.5 µg/ml G-CSF + 50 µg/ml anti-G-CSF Ab | 4373.0 ± 278.1 |
| **Anti-G-CSF Ab administration** | \(^{[3]}\)H]thymidine incorporation (cpm/well) | |
| Control | 4132.2 ± 231.4 |
| 10 µg/ml anti-G-CSF Ab | 3750.8 ± 178.8 |
| 50 µg/ml anti-G-CSF Ab | 3326.0 ± 246.2 |
| 200 µg/ml anti-G-CSF Ab | 3166.7 ± 113.0 |

*The uptake at 24-h incubation after the final G-CSF administrations of 0.1 and 0.5 µg/ml were 5344.7 ± 680.1 and 6030.8 ± 409.5 cpm, respectively; these were significantly higher than those in the controls (4760.6 ± 309.1 cpm; \(P < 0.05\)). When 0.5 µg/ml G-CSF was preincubated with serial concentrations of anti-G-CSF antibody, \(^{[3]}\)H]thymidine incorporation was 6180.8 ± 285.3, 4849.8 ± 216.5, and 4373.0 ± 278.1 cpm at 0, 10, and 50 µg/ml of anti-G-CSF antibody, respectively. \(^{[3]}\)H]thymidine incorporation in cultures with anti-G-CSF antibody was statistically significantly lower than was that in cultures without anti-G-CSF antibody (\(P < 0.01\)). Furthermore, when anti-G-CSF antibody was added in the cultures every 24 h for three times, \(^{[3]}\)H]thymidine incorporation was 3750.8 ± 178.8 cpm for 10 µg/ml, 3326.0 ± 246.2 cpm for 50 µg/ml, and 3166.7 ± 113.0 cpm for 200 µg/ml of anti-G-CSF antibody. These values were significantly lower than were those without anti-G-CSF antibody administration (4132.2 ± 231.4 cpm/well; \(P < 0.01\)); Ab, antibody.
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