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Granulocyte Colony Stimulating Factor (GCSF) Alters the Phenotype of Neuroblastoma Cells: Implications for disease free survival of high-risk patients

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Abstract

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Introduction—GCSF is commonly employed for the treatment of chemotherapy-induced neutropenia. Despite high-dose intensive chemotherapy for advanced stage neuroblastoma, the survival rate remains poor. GCSF therapy is quite common in these children, thus we questioned its effect on neuroblastoma cells. We hypothesized that exogenous GCSF stimulates the proliferation and invasive character of neuroblastoma cells.

Methods—Expression of a GCSF receptor in five different neuroblastoma cell lines was determined by PCR. In addition, we determined the effect of increasing doses of GCSF (0, 1 ng/ml, 10 ng/ml, 1 µg/ml, 10 µg/ml) on DNA synthesis (BrdU assay), invasiveness (Matrigel invasion chambers) and cell proliferation.

Results—We tested five neuroblastoma cell lines; all expressed the GCSF receptor. GCSF treatment resulted in significantly increased proliferation of SK-N-SH, SK-N-AS and SHSY-5Y cells. Likewise, increased invasiveness of SK-N-SH cells was observed with GCSF treatment.

Conclusions—Our results indicate that neuroblastoma cell lines express the GCSF receptor and respond to exogenous GCSF by increased proliferation and invasiveness. These findings suggest that GCSF may stimulate the growth of neuroblastoma cells in patients undergoing high-dose chemotherapy with GCSF rescue and could have a significant impact on the ability to eradicate these tumors.

Keywords

GCSF; neuroblastoma; recurrence; GCSF-receptor; cytokine; childhood tumor

INTRODUCTION

The current therapies for high-risk neuroblastoma include dose-intensive induction chemotherapy to induce tumor remission, followed by myeloablative chemotherapy with autologous marrow or peripheral blood stem cell rescue [1–3]. Granulocyte colony stimulating factor (GCSF, CSF3) is often administered in hope of restoring neutrophil levels after myeloablative chemotherapy in these neuroblastoma patients [4]. GCSF is also employed to stimulate leukocytosis just before peripheral blood stem cell collection in high-risk neuroblastoma [5–7]. Despite these dose-intensified chemotherapeutic regimens, less than half of high-risk neuroblastoma patients who receive current therapy were alive and free of disease at five years [1,8]. These trends suggest the possibility that current chemotherapy drugs alone cannot eradicate the most aggressive neuroblastomas. It raises the possibility that other factors in the tumor milieu are contributing to tumor survival or reconstitution.

GCSF, along with other growth factors, has been documented to promote multiple human cancers. Specifically, GCSF has been associated with tumor progression, increased invasiveness and poorer prognosis in tumors such as head and neck squamous cell carcinoma [9], non-small cell lung cancer [10,11], bladder [12] and prostate cancer [13]. The reported association between GCSF, GCSF receptors and other tumors led us to question whether GCSF has an effect on neuroblastoma tumor growth. We therefore proposed the hypothesis that the poor response seen in patients with high-risk neuroblastoma is due to “rescue” of neuroblastoma cells with GCSF therapy. To test this hypothesis, we investigated the expression of the GCSF receptor on neuroblastoma cell lines and the effect of exogenous GCSF on cell proliferation and invasiveness.

METHODS

Cell culture

Five neuroblastoma cell lines, LAN-1, SHSY-5Y, SK-N-AS, IMR-32 and SK-N-SH (American Type Culture Collection, Manassas, VA) were cultured in either modified Eagle's medium (MEM) or Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/ml streptomycin/penicillin (GIBCO, Grand Island, NY, USA). All cells were incubated at 37°C in 100% humidity with 5% CO₂.

Detection of GCSF receptor mRNA

The expression of GCSF-receptor mRNA in neuroblastoma cells was determined using previously described techniques [12]. Briefly, five neuroblastoma cell lines (LAN-1, SHSY-5Y, SK-N-AS, IMR-32 and SK-N-SH) were grown in culture to 50 – 75% confluency. Ribonucleic acid (RNA) was isolated from the neuroblastoma cell lines using standard techniques. RNA isolated from human neutrophils of a healthy volunteer served as the control. The RNA concentration was determined by spectrophotometry.

Reverse transcription polymerase chain reaction was performed as described previously [12]. The integrity of cDNA was confirmed by electrophoresis and ultraviolet light. Amplification of cDNA was then performed as described previously [12] using the forward primer sequence: TGCCCGGCCTTGGCAGCCACCCATC; and reverse primer sequence: TGGGGCTGCCAGCAGCTGCCATA. After amplification heating from 95° C to 60° C to 95° C over 1 min was performed to allow melting curve analysis. The samples were then electrophoresed on 1% agarose gel for 1 hour at 80 millivolts, stained with ethidium bromide and photographed under ultraviolet light. The reaction produced a 225 base-pair band.

DNA synthesis assay

SK-N-SH cell line was maintained in tissue culture (DMEM with 5% fetal bovine serum). Upon attaining 50% confluency, 3 ml of fresh media was added containing either 0, 10 ng/ml, 100 ng/ml, 1 µg/ml, or 10 µg/ml of recombinant human GCSF (Amgen Inc., Thousand Oaks, CA). Bromodeoxyuridine (BrdU) 10 µM was added to the culture medium per manufacturer's instructions (Roche, FLUOS in situ cell proliferation kit, Nutley NJ, USA). The cells were incubated at 37° C for 24 hrs, trypsinized, washed with phosphate-buffered saline (PBS) and centrifuged. The pellet was resuspended in 1 ml ethanol fixative at 4° C for 30 min, washed in PBS and resuspended in 0.5 ml HCl denaturation solution. The cells were then washed again and suspended in fluorescein-conjugated anti-BrdU and incubated for 45 min. at 37° C. The antibody-labeled cells were washed, centrifuged, resuspended in 0.5 ml PBS and counted with a flow cytometer (Becton Dickinson, FACscan, Franklin Lakes, NJ, USA). (n=5)

Cell proliferation assay

As DNA synthesis may not necessarily reflect cell proliferation in neoplastic cells, we also measured viable cell numbers after growth with GCSF present in culture medium. Cells were plated onto 96-well plates at a concentration of 5×10^3 cells/well. GCSF was added to culture medium to a final concentrations of 0 (pure DMEM added), 10 ng/ml, and 100 ng/ml two days after initial plating, when cells were adherent to the bottom of the well. Using the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD), cell proliferation was determined on days 1, 3, 5 and 7 following GCSF administration. Cell Counting Kit-8 tetrazolium salt reagent was added to the culture medium. After a 2 hour incubation period, optical density (O.D.) was measured at 450nm using a standard plate reader (ELx808 Ultra Microplate Reader, Bio-Tek Instruments, Inc.). All experiments were carried out in triplicate.

The fold increase in cell number compared to baseline values was expressed as mean $\pm$ standard deviation reported.

Invasiveness assay

To determine how GCSF affects the invasive potential of neuroblastoma cells, we used an in-vitro Matrigel invasion chamber. SK-N-SH cells were grown in DMEM (5% fetal bovine serum) to 80–90% confluency, trypsinized, and counted. Aliquots of 15,000 cells were plated onto Matrigel invasion chambers (Becton Dickinson Biosciences). The supernatant from a 3T3 fibroblast cell line (American Type Culture Collection, Manassas, VA), known for naturally high concentration of chemoattractants, was used as the chemoattractant on the opposite side of invasion of the matrigel. GCSF was added to the growth medium in separate wells at concentrations of 0, 10 ng/ml, 100 ng/ml, 1 μ g/ml, or 10 μ g/ml. The cells were incubated for 22 hrs. Matrigels were removed and stained with Wright-Giemsa. A blinded observer counted number of invasive cells per field (5 fields) assisted by a scored reticular eyepiece. Four separate experiments were performed, each carried out in duplicate.

Statistical analysis

Values are expressed as mean $\pm$ standard deviation. Student's t-test and ANOVA were used as appropriate with Tukey post-hoc analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Neuroblastoma cell lines express GCSF receptor mRNA

All five neuroblastoma cell lines tested expressed the expected 225 base pair band reaction product consistent with the GCSF receptor (Figure 1). This finding suggests that these neuroblastoma cells are capable of responding to exogenous GCSF.

Increased DNA synthesis by GCSF

GCSF treatment resulted in increased DNA synthesis by SK-N-SH cells at pharmacologic doses (10 ng/ml, 100 ng/ml) (figure 2). BrdU incorporation is noted in cells undergoing the S-phase of cell cycle. In groups not treated with GCSF, an average of $4.7\% \pm 0.3$ of the cells were in S phase. In contrast, statistically significantly more cells were in S-phase when the cells were treated with either 10 ng/ml GCSF ($13.3\% \pm 4.1$) or 100 ng/ml GCSF ($13.5\% \pm 5.4$). However, higher doses of GCSF exhibited wide variation in DNA synthesis that was not statistically different from untreated cells. In children ages 1–9 undergoing myelosuppressive chemotherapy for neuroblastoma, typical serum concentration 4–12 hours after 5–15 μ g/kg subcutaneous administration of GCSF is 3–117 ng/ml [14]. Thus, GCSF can increase DNA synthesis of neuroblastoma cells at doses used in the clinical setting.

GCSF increases neuroblastoma cell proliferation

SK-N-SH cells grown in culture medium supplemented with GCSF at 10 ng/ml or 100 ng/ml exhibited statistically higher viable cell counts after 3, 5, and 7 days of growth ($p < 0.01$) (Figure 3). This ability of GCSF to promote proliferation was also noted in SK-N-AS and SHSY-5Y neuroblastoma cell lines. Interestingly, SK-N-AS cells exhibited significantly higher viable cell counts when grown for 3 and 5 days with both 10 ng/ml and 100 ng/ml ($p < 0.01$) but this effect was lost at day 7 (Figure 4). Rather, a statistically significant decrease in cell proliferation was noted by SK-N-AS cells on day 7 at the highest concentration of GCSF (100 ng/ml). SHSY-5Y cells also exhibited higher cell counts on days 3, 5 and 7 when treated with GCSF compared to untreated controls (Figure 5) although fewer cells were counted on day 7 in the GCSF 100 ng/ml group compared to the GCSF 10 ng/ml group. The reason for this finding is

not determined in this study, but may be due to inhibition of proliferation of GCSF at sustained high doses.

GCSF stimulates increased invasiveness of neuroblastoma cells

A well-established method of assessing invasive nature of tumor cells in-vitro was employed [12]. When exposed to varying doses of GCSF, SK-N-SH cells demonstrated a dose-dependent increase in their ability to invade matrix with a peak response at 1 μ g/ml. However, even at the pharmacologic range of 100 ng/ml, GCSF resulted in a significant increase in the invasiveness of SK-N-SH cells compared to untreated controls.

DISCUSSION

We embarked upon this study to determine whether neuroblastoma cells express the GCSF-receptor and if GCSF increases proliferation and invasiveness of neuroblastoma cells. Our results indicate that neuroblastoma cell lines do express the mRNA for the GCSF-receptor (Figure 1) and respond to exogenous GCSF stimulation. The propensity for tumor cells to respond to growth factors is readily observed in several types of cancer [9–13]. This documented response to growth factors, coupled with the observation that neuronal progenitor cells utilize GCSF as a growth and development factor in normal development [15] suggest that these cells either never lose GCSF receptor expression, or reclaim expression as part of functional changes during mutagenesis or oncogenesis [16].

Our observation that exogenous GCSF stimulation results in increased DNA synthesis is contrary to the findings of Morel et. al.[17]. We demonstrated increase in DNA synthesis at 24 hours (Figure 2) and increase in total cell count (Figure 3, Figure 4 and Figure 5). We made our observations in non-confluent cells at early time points to accurately assess the growth phase of the cells. Furthermore, our observations are noted at GCSF concentrations similar to serum concentrations of 3–117 ng/mL observed 4–12 hours after subcutaneous administration of 5–15 μ g/kg of GCSF in children [14]. The increase cell count following GCSF treatment may be due to increased cell division or inhibition of apoptosis. The anti-apoptotic gene NOR3 has been observed to be upregulated in response to GCSF in the SHSY-5Y neuroblastoma cell line [17]. Our results indicate that DNA synthesis and cell count are increased following GCSF stimulation. Thus, both cell division and inhibition of apoptosis appear to play a role.

Tumor invasion and metastasis are features of advanced and aggressive tumors. Our results indicate that GCSF stimulation results in increased invasiveness of neuroblastoma cells (Figure 5). Invasion through Matrigel implies upregulation of anchoring molecules such as integrins and expression of collagenases, which has been documented in bladder cancer cell lines [12]. Although an artificial system, the mechanics required for Matrigel invasion are similar to those needed for tumor invasion in-vivo. GCSF therapy could therefore also result in increased invasiveness and metastasis of neuroblastoma cells in-vivo.

The effectiveness of GCSF in resolving neutropenia is not in question. While resolution of neutropenia and thrombocytopenia allows for earlier resumption of the chemotherapeutic regimen, our study suggests that the potential beneficial effects of GCSF on recovery of myeloid function may be negated by its direct stimulation of the tumor cells.

This study is limited by the use of immortalized cell lines. Our findings would need to be correlated with primary neuroblastoma cells. In addition, the expression of GCSF receptor is inferred by mRNA expression and not demonstrated by expression of the surface protein. However, we have shown that exogenous GCSF stimulates these neuroblastoma cells. Our findings provide preliminary data and adequate justification to further assess the effects of GCSF in the treatment and outcomes of children with high-risk neuroblastoma.

This study shows that neuroblastoma cells express the GCSF receptor and are stimulated by exogenous GCSF. These findings suggest that GCSF may stimulate the growth of neuroblastoma cells in patients undergoing high dose chemotherapy with G-CSF rescue and could have significant impact on the ability to eradicate these tumors.

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REFERENCES

1. Matthay KK, Villablanca JG, Seeger RC, et al. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. *N Engl J Med* 1999;341:1165–1173. [PubMed: 10519894]
2. Cheung NV, Heller G. Chemotherapy dose intensity correlates strongly with response, median survival, and median progression-free survival in metastatic neuroblastoma. *J Clin Oncol* 1991;9:1050–1058. [PubMed: 2033419]
3. Grupp SA, Stern JW, Bunin N, et al. Tandem high-dose therapy in rapid sequence for children with high-risk neuroblastoma. *J Clin Oncol* 2000;18:2567–2575. [PubMed: 10893288]
4. Michon JM, Hartmann O, Bouffet E, et al. An open-label, multicentre, randomised phase 2 study of recombinant human granulocyte colony-stimulating factor (filgrastim) as an adjunct to combination chemotherapy in paediatric patients with metastatic neuroblastoma. *Eur J Cancer* 1998;34:1063–1069. [PubMed: 9849455]
5. Kanold J, Rapatel C, Berger M, et al. Use of G-CSF alone to mobilize peripheral blood stem cells for collection from children. *Br J Haematol* 1994;88:633–635. [PubMed: 7529535]
6. Schroeder H, Kamperis K, Grunnet N, et al. Prediction of target CD34 positive cells following leukopheresis in children with neuroblastoma. *Pediatr Blood Cancer* 2006;46:786–792. [PubMed: 16333829]
7. Kletzel M, Katzenstein HM, Haut PR, et al. Treatment of high-risk neuroblastoma with triple-tandem high-dose therapy and stem-cell rescue: results of the Chicago Pilot II Study. *J Clin Oncol* 2002;20:2284–2292. [PubMed: 11980999]
8. George RE, Li S, Medeiros-Nancarrow C, et al. High-risk neuroblastoma treated with tandem autologous peripheral-blood stem cell-supported transplantation: long-term survival update. *J Clin Oncol* 2006;24:2891–2896. [PubMed: 16782928]
9. Gutschalk CM, Herold-Mende CC, Fusenig NE, et al. Granulocyte Colony-Stimulating Factor and Granulocyte-Macrophage Colony-Stimulating Factor Promote Malignant Growth of Cells from Head and Neck Squamous Cell Carcinomas In vivo. *Cancer Res* 2006;66:8026–8036. [PubMed: 16912178]
10. Mroczko B, Szmitkowski M, Niklinski J. Granulocyte-Colony stimulating factor and macrophage-colony stimulating factor in patients with non-small-cell lung cancer. *Clin Chem Lab Med* 2001;39:374–379. [PubMed: 11434384]
11. Nakata H, Uemura Y, Kobayashi M, et al. Cyclooxygenase-2 inhibitor NS-398 suppresses cell growth and constitutive production of granulocyte-colony stimulating factor and granulocyte macrophage-colony stimulating factor in lung cancer cells. *Cancer Sci* 2003;94:173–180. [PubMed: 12708493]
12. Chakraborty A, White SM, Lerner SP. Granulocyte colony-stimulating factor receptor signals for beta 1-integrin expression and adhesion in bladder cancer. *Urology* 2004;63:177–183. [PubMed: 14751388]
13. Pirtskhalaishvili G, Nelson JB. Endothelium-derived factors as paracrine mediators of prostate cancer progression. *Prostate* 2000;44:77–87. [PubMed: 10861760]
14. Stute N, Santana VM, Rodman JH, et al. Pharmacokinetics of subcutaneous recombinant human granulocyte colony-stimulating factor in children. *Blood* 1992;79:2849–2854. [PubMed: 1375115]
15. Schneider A, Kruger C, Steigleder T, et al. The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. *J Clin Invest* 2005;115:2083–2098. [PubMed: 16007267]

16. Nitta T, Sato K, Allegretta M, et al. Expression of granulocyte colony stimulating factor and granulocyte-macrophage colony stimulating factor genes in human astrocytoma cell lines and in glioma specimens. *Brain Res* 1992;571:19–25. [PubMed: 1377084]
17. Morel M, Beaujean F, Le Forestier C, et al. Effect of human granulocyte colony stimulating factor on neuroblastoma cell growth. *Biomed Pharmacother* 1993;47:29–32. [PubMed: 7687155]

Figure 1.
Gel demonstrating a 225 base pair PCR product prominent in all neuroblastoma cell lines tested as well as human neutrophils (positive control).

Figure 2. SK-N-SH DNA synthesis. There is an increase in DNA synthesis as demonstrated by incorporation of BrdU at all doses of GCSF tested. However, this is only statistically significant at 10 and 100 ng/ml.

Figure 3.

SK-N-SH cell proliferation assay: There is a progressive increase in cell proliferation over the duration of culture. GCSF treated cells demonstrated a significantly higher increase in the rate of proliferation, attaining a 10 fold increase from baseline in the GCSF-treated group compared to merely an 8-fold increase in the untreated group.

Figure 4.

SK-N-AS Cell proliferation assay: Compared to untreated controls, a significant increase in cell proliferation is observed at 3 and 5 days post GCSF treatment with both 10 and 100 ng/ml. At 7 days post treatment, a significant decrease in proliferation is noted in the 100 ng/ml treatment group.

Figure 5.

SKSY-5Y cell proliferation assay: Increased proliferation of GCSF treated cells is noted at days 3, 5, and 7 days post treatment compared to untreated controls.

Figure 6.

Invasive potential of SK-N-SH cells in response to GCSF: GCSF treatment resulted in an increase in invasiveness of SK-N-SH cells at all doses tested with a peak effect at 1 µg/ml.