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Paradoxical Drop in Circulating Neutrophil Count Following Granulocyte-Colony Stimulating Factor and Stem Cell Factor Administration in Rhesus Macaques:

Rapid Neutrophil Decline Following G-CSF Administration Cytokines

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Abstract

Objective—Granulocyte colony stimulating factor (G-CSF) is frequently used therapeutically to treat chronic or transient neutropenia and to mobilize hematopoietic stem cells. Shortly following G-CSF administration, we observed a dramatic drop in circulating neutrophil number. This paper characterizes this effect in a rhesus macaque animal model.

Methods—Hematologic changes were monitored following subcutaneous(SQ) administration of G-CSF. G-CSF was administered as a single SQ dose at 10µg/kg or 50µg/kg. It was also administered (10µg/kg) either alone or in combination with SCF (200µg/kg) over five days. Flow cytometry was performed on serial blood samples to detect changes in cell surface adhesion protein expression.

Results—Neutrophil count dramatically declined 30 minutes following G-CSF administration. This decline was observed whether 10µg/kg G-CSF was administered alone or in combination with SCF over five days, or given as a single 10µg/kg dose. At a single 50µg/kg dose the decline accelerated to 15 minutes. Neutrophil count returned to baseline after 120 minutes and rapidly increased thereafter. An increase in CD11a and CD49d expression coincided with the drop in neutrophil count.

Conclusion—A paradoxical decline in neutrophil count was observed following the administration of G-CSF either alone or in combination with SCF. This decline accelerated with the administration of a higher dose of G-CSF and was associated with an increase in CD11a and CD49d expression. It remains to be determined whether this decline in circulating neutrophils is associated with an increase in endothelial margination and/or entrance into extravascular compartments.

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INTRODUCTION

G-CSF was molecularly cloned 20 years ago [1–3]. It stimulates the production, release and redistribution of mature neutrophils [4–8], and also plays a role in the regulation of neutrophil behavior [9]. The first clinical trials of G-CSF in humans targeted the treatment of chronic neutropenias (cyclic, congenital, and idiopathic) [10–13]. G-CSF therapy has also been used to accelerate neutrophil recovery following hematopoietic cell transplantation [14]. This hematopoietic growth factor has become the standard of care for the treatment of patients with genetic and chemotherapy-related neutropenia [4–6,8,14,15], and is regularly used to mobilize hematopoietic stem and progenitor cells into circulation so that they can easily be collected by leukapheresis for transplantation [7,16,17]. SCF has been molecularly cloned [18–20] and synergistically augments mobilization when given in combination with G-CSF [7,16,17,21]. The combination of G-CSF and SCF is regularly used in non-human primates (NHPs) to maximize the number of CD34⁺ hematopoietic stem cells in circulation [22]. SCF is not currently being used clinically in humans due to its potential to cause anaphylactic reactions [23].

Shortly following the administration of G-CSF alone or in combination with SCF in NHPs there is a paradoxical decline in neutrophil count. Within two hours, the neutrophil count recovers and surpasses pre-administration levels. This neutrophil disappearance has been previously observed in mice, rats, and humans [4–6,15,24,25]. To better understand this phenomenon, neutrophil counts were followed at multiple time points following cytokine administration in NHPs. Flow cytometric analysis was used to evaluate changes in cell surface protein expression. Proteins monitored include CD11a (LFA-1), CD18 (VAP-1), CD31 (PECAM-1), CD44 (H-CAM), CD49d (VLA-4), CD62L (LAM-1, L-selectin) and CD184 (CXCR4).

METHODS

Administration of Cytokines to Rhesus Macaques

All animals were housed and handled in accordance with the guidelines set forth by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [26]. Rhesus macaques (*Macaca mulatta*) used in this study were specific pathogen free males and females between 4–6 years of age and seronegative for the Herpes B virus, the simian immunodeficiency virus, the simian T-cell leukemia virus, and the simian retrovirus Type-D. A total of seven animals were evaluated following a single dose of G-CSF (Filgrastim, kindly provided by Amgen, Inc., Thousand Oaks, CA) administered subcutaneously (SQ) at a dose of either 10µg/kg or 50µg/kg. All animals were given a minimum of four weeks to recover before undergoing re-mobilization with the alternative cytokine dose regimen. Four animals were administered SQ a total of five doses of G-CSF once daily (10µg/kg/d) for five consecutive days either alone or in combination with SCF (200µg/kg/d). Recombinant human SCF was also kindly provided by Amgen, Inc. EDTA anti-coagulated samples were taken prior to and subsequent to cytokine administration for automated complete and differential blood cell counts using a Cell Dyn 3500 (Abbott Diagnostics, Santa Clara, CA). Based on a sampling of 100 non-cytokine mobilized rhesus macaques, normal white blood cell (WBC) counts range from 3,800 to 12,200 WBC/µl with a 20–56% neutrophil differential.

Animals were sedated daily for blood collection using a combination of Ketamine HCl and Telazol (3mg/kg each) as an intramuscular (IM) injection. The animals were intubated and maintained under 1–2% isoflurane anesthesia through the 120 minute sample. Ketamine alone at a dose of approximately 5 mg/kg IM was used to collect the 180 and 240 minute samples. A 1–2ml EDTA sample was taken prior to the injection of cytokines (pre-sample).

The pre-sample was used as the baseline for determining changes that occurred post-cytokine administration. Subsequent 1–2ml EDTA samples were collected at 15, 30, 45, 60, 90, 120, 180, 240 minutes, and 24 hours post-injection. Forty-five minute and 180 minute time-points were not taken during the single dose experiment. Animals were administered iron folate (10mg/kg IM), folic acid (0.25mg/kg IM), and Metacam (0.20 mg/kg SQ) following the collection of the 240 minute blood sample.

Flow Cytometry

EDTA anti-coagulated samples were centrifuged and 1ml of plasma was removed. This volume was replaced with an equal volume of PBS containing 400µg/ml of Chromepure mouse IgG (Jackson ImmunoResearch, West Grove, PA) to block any non-specific binding to Fc receptors. The tubes were vortexed and incubated for 10 minutes in the dark. Either isotype controls or antibodies were added to 100µl of blocked whole blood. All antibodies were titrated and confirmed to provide specific binding in rhesus macaques prior to the initiation of the study. 0.06µg IgG1 APC (BD Pharmingen, San Diego, CA), 0.25µg IgG1 PE (BD Pharmingen), 0.5µg IgG1 FITC (BD Pharmingen), 1.0µg IgG2a PE (BD Pharmingen), 1.0µg IgG2a PE-Cy7 (Caltag Laboratories, Burlingame, CA), 0.5µg IgG2b FITC (Caltag Laboratories), 0.06µg CD11a APC (BD Pharmingen), 0.25µg CD18 PE (BD Pharmingen), 1.0µg CD31 FITC (BD Pharmingen), 0.5µg CD44 FITC (Caltag Laboratories), 0.125µg CD49d FITC (Beckman Coulter, Fullerton, CA), 1.0µg CD62L PE (BD Pharmingen), 0.5µg CD184 PE (BD Pharmingen) and 0.25µg CD184 PE-Cy7 (eBiosciences, San Diego, CA) were added to the appropriate tubes and stained according to the manufacturer's instructions. Samples were incubated in the dark for 20 minutes. Q-Prep (Beckman Coulter, Fullerton, CA) was used to rinse the cells, lyse red blood cells, and fix samples. Cells only, isotype, and antibody stained samples were run using an FC500 flow cytometer (Beckman Coulter, Miami, FL).

Analysis

Flow results were analyzed with Cytomics RXP Analysis Software (Beckman Coulter, Miami, FL). The granulocyte population was analyzed by gating on forward (size) and side (granularity) scatter. 25,000 events were evaluated within the gated granulocyte population at each time-point (approximately 10,000 events were evaluated at 15 and 30 minutes due to decline in neutrophil numbers). Mean fluorescent intensity (MFI) was calculated by the Cytomics RXP Analysis Software. Statistical tests were determined using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA) and Microsoft Office Excel (Microsoft Corporation, Bellevue, WA).

RESULTS

Single Dose Administration of G-CSF

Seven animals were evaluated following a single SQ dose of 10µg/kg and 50µg/kg G-CSF. Experiments at the two dose levels were performed at least four weeks apart. All mobilizations demonstrated a decline in circulating neutrophils shortly after administration (Fig 1a and b). Neutrophil counts reached a nadir at 30 minutes following cytokine administration at the 10µg/kg dose and returned to baseline levels 120 minutes post-administration (Fig 1a). Administering G-CSF at the higher dose of 50µg/kg resulted in an accelerated neutrophil decline (Fig 1b). The observed decline for this test group occurred at 15 minutes post-injection, following which the neutrophil count achieved and then surpassed the baseline within 120 minutes post-administration.

Multiple Dose Administration of G-CSF

Animals in the multiple dose condition were mobilized over a five day period with either G-CSF alone (10µg/kg/day SQ) or a combination of G-CSF (10µg/kg/day SQ) and SCF (200µg/kg/day SQ). Experiments using the two cytokine conditions were performed at least four weeks apart. All mobilizations demonstrated a decline in neutrophil number following the final administration of cytokines. Similar to the 10µg/kg single dose G-CSF study, the decline in neutrophil count occurred at the 30 minute time point whether G-CSF was used alone or in combination with SCF (Fig 1c).

Flow Cytometric Analysis of Single Dose Administration

Flow cytometric data showed a significant up-regulation of several cell surface adhesion molecules during the period of decline in neutrophil count. Increases in MFI were observed at 30 minutes for CD11a and CD49d at the 10µg/kg dose of G-CSF (Fig 2). This effect carried over to the 50µg/kg dose group, which expressed the highest levels of CD11a and CD49d at the nadir point of 15 minutes (Fig 2). *In vitro* studies using 1×10^6 Ficoll™ purified granulocytes from naïve animals when treated with 50ng/ml of G-CSF failed to demonstrate any upregulation of adhesion proteins at 30 and 240 minutes when compared to granulocytes maintained in media alone (data not shown).

Flow Cytometric Analysis Multiple Dose Condition

A distinct subpopulation of granulocytes showed increased expression of CD11a, CD18, CD31, and CD49d. These “bright” sub-populations were defined as having an approximately 5-fold increase in mean fluorescent intensity (Fig 3). Peak cell adhesion protein expression occurred 30 minutes post cytokine administration, coinciding with the circulating neutrophil nadir (Fig 4). Protein expression returned to baseline within 180 minutes post-cytokine administration. CD44, CD62L, and CD184 demonstrated negligible or inconsistent changes in expression (data not shown).

DISCUSSION

G-CSF is widely used clinically to treat genetic and therapy associated neutropenia and to induce mobilization of hematopoietic progenitors and stem cells into circulation. Kinetic studies in mice, rats, and humans have noted a transient decline in neutrophil count following G-CSF administration [4–6,15,24,25]. We observed a similar phenomenon in NHPs when G-CSF was administered either alone or in combination with SCF. The purpose of this study was to explore this phenomenon further.

Previous studies have noted that G-CSF can induce rapid changes in cell surface protein expression and cellular activation [4,6,27–29]. Our results suggest that G-CSF alone or in combination with SCF can elicit a significant drop in circulating neutrophil number shortly after its administration. This decline was associated with an increase in adhesion protein expression in all test groups. This association may be causative or simply related to a proportional change in cell population following neutrophil loss. The increase seen in adhesion protein expression could be due to a release of cell adhesion receptors from a pre-formed intracellular pool or induction of gene expression and the formation of new proteins. Neutrophils having higher levels of G-CSF receptors may be more responsive than those with fewer receptors. Although upregulation of cell surface adhesion proteins may play a role in neutrophil loss, other mechanisms can be involved as well. For example, G-CSF may directly enhance the avidity of adhesion receptors on neutrophils to endothelium, or G-CSF may have an indirect response on other cells by releasing proteins that would promote neutrophil-endothelial interactions. Studies performed *in vitro* using purified neutrophils from naïve animals failed to demonstrate an increase in adhesion protein expression when

exposed to G-CSF. This suggests that the observed neutrophil decline may be due to an indirect response, such as a transient release of a granulocyte associated protein that would enhance neutrophil-endothelial cell adhesion. The molecular mechanism for the loss and the ultimate fate of the disappearing neutrophils has yet to be determined. Is there, for example, a transient splenic sequestration and release of neutrophils and/or do the neutrophils marginate, enter extravascular compartments, and then 'new' neutrophils generated? Such questions will require careful molecular imaging studies to be answered.

The rate of neutrophil disappearance was found to be G-CSF dose dependent. At a SQ dose of 50µg/kg, neutrophil numbers were diminished at 15 minutes rather than the 30 minutes observed when 10µg/kg was used (Fig 1a versus Fig 1b). Using flow cytometric analysis a correlation again was observed between neutrophil loss and an increase in cell adhesion protein MFI for CD11a and CD49d (Fig 2).

Previous observations of changes in cell surface protein expression *in vitro* and *in vivo* include an increase in CD62L [4], CD11b [4,6,27,30–33], CD18 [30–33], CD14, CD32 and CD64 [9,30]. The increase in CD62L is controversial as others have observed a decrease in CD62L expression [4,15,32,34], or an increase in the ligand affinity of CD62L (without changes in cell surface expression) [6]. CD11a, CD49d, and CD18 are all members of the integrin protein family (α -integrin, α -, and β -, respectively). CD11a and CD18 are found in association with each other, and together function as receptors for ICAM-1, 3 (CD54, CD50), and a ligand for VAP-1 (CD102). It has been noted that changes in neutrophil count and protein expression may in part be due to an increase in the functional capacity of β -1 and β -2 integrin proteins [33]. Therefore, not only changes in surface adhesion protein expression may be responsible for neutrophil loss, but functional changes in receptor avidity may also play a role. Each of these options should be considered in future studies.

Investigation of the characteristics of neutrophils in dysfunctional hematopoietic systems can help elucidate neutrophil function. Studies of patients with advanced liver disease and myelodysplastic syndromes show that their neutrophils display an increased expression of CD11b and a decreased expression of CD62L when compared with healthy controls [27,28]. Changes in neutrophil behavior in these patients include increased cellular adhesion but decreased trans-endothelial migration. In patients with liver disease treated with G-CSF there is an improvement in neutrophil trans-endothelial migration, while cellular adhesion was unaffected [27]. Like patients with chronic neutropenia and immunosuppression [10–13], patients with advanced liver disease and myelodysplastic syndromes have a significantly increased susceptibility to infections. The relative inability of their neutrophils to cross the endothelial barrier may seriously compromise the body's ability to combat infection [27,28]. These findings suggest that G-CSF may play a role in the migration of neutrophils into extravascular compartments. This may help in explaining the rapid loss of neutrophils within the circulation following G-CSF administration.

Our study provides evidence that there is a rapid loss in neutrophil number following G-CSF administration. The mechanism for this loss remains a puzzle. It may be associated with an increase in adhesion protein expression, but other mechanisms may be involved as well. Whether these neutrophils marginate and/or enter extravascular compartments will require careful imaging studies. It is interesting to note that a significant increase in spleen size has been observed in humans during the course of G-CSF administration [10,12,13,35]. As imaging technology improves, such questions may be more effectively addressed using the NHP model system. Further studies are needed in order to ascertain the molecular mechanism and clinical relevance behind this paradoxical decline in circulating neutrophils.

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Figure 1a: **Figure 1b:** **Figure 1c:** **Figure 1.**

Figure 1a: Changes in mean circulating neutrophil count and standard error following a single dose of subcutaneous G-CSF at 10 µg/kg (n=6).

Figure 1b: Changes in mean circulating neutrophil count and standard error following a single dose of subcutaneous G-CSF at 50 µg/kg (n=6).

Figure 1c: Changes in mean circulating neutrophil count and standard error following the 5th dose of subcutaneous G-CSF (n=4) or G-CSF + SCF (n=4).

Figure 2.
Changes in the Mean Fluorescence Intensity and the standard error for CD11a and CD49d expression following single doses of G-CSF at 10µg/kg (n=6) and 50µg/kg (n=6).

Figure 3.
Examples of CD11a, CD18, CD31, and CD49d expression following G-CSF and SCF administration.

Figure 4.
Mean fold changes and standard error for CD11a, CD18, CD31 and CD49d expression following G-CSF (n=4) or G-CSF+SCF administration (n=4).