Granulocyte-Macrophage Colony-Stimulating Factor Induces the Expression of the CD11b Surface Adhesion Molecule on Human Granulocytes in Vivo

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The CD11b (Mol) molecule is a member of a family of surface glycoproteins that are essential for adhesion-dependent granulocyte functions. Brief exposure of granulocytes to human granulocyte-macrophage colony-stimulating factor (GM-CSF) in vitro increases the surface expression of CD11b and increases granulocyte adhesiveness. To assess the possible in vivo significance of these observations we studied the effect of GM-CSF on CD11b, CD11a (LFA-1), and CD11c (gp 150, 95) expression on granulocytes from nine adult patients with sarcoma who were receiving GM-CSF as part of a phase I trial. GM-CSF was administered as a continuous infusion at a dose of 32 or 64 µg/kg/d. Granulocyte CD11b, CD11a, and CD11c expression was determined by indirect immunofluorescence staining of whole blood, thereby minimizing in vitro manipulation. A transient leukopenia developed within 15 minutes of initiation of GM-CSF treatment that was associated with a marked increase in the surface antigen density of CD11b. A mean 1.7-fold increase in the percentage of CD11b-positive granulocytes and a mean 2.1-fold increase in CD11b surface antigen density was noted after 12 hours of treatment. No change in CD11a or CD11c expression was observed over the first 12 hours. The level of CD11b expression was followed in six patients for up to 5 days of treatment with GM-CSF. Compared with the 12-hour value, three of six patients showed a subsequent decrease in CD11b expression, two remained constant, and one showed a continued increase in CD11b surface density. Fluorescence-activated cell sorting of granulocytes into high- and low-density CD11b-positive groups revealed a preponderance of immature myeloid forms in the low-density CD11b fraction, which suggests that the late decrease in CD11b expression in some patients may be related to a greater proportion of circulating immature myeloid forms in the peripheral blood. This study suggests that GM-CSF administered as a continuous infusion rapidly upregulates the expression of granulocyte CD11b in vivo. The influence of this phenomenon on in vivo granulocyte aggregation may be clinically relevant with regard to the toxicity of GM-CSF and deserves further investigation.

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volunteers donating blood for some experiments also gave informed consent.

Administration of GM-CSF. Patients received purified rGM-CSF (Sandoz Research Institute, East Hanover, NJ) by continuous infusion through a central venous catheter on a protocol assessing the effects of GM-CSF on chemotherapy-induced myelosuppression. The GM-CSF was administered in a preservative-free normal saline solution. The specific activity of the material used in this study was 8.0 x 10^6 U/mg of protein as determined in vitro in a rapid-proliferation assay that measures incorporation of [3H] thymidine by GM-CSF-responsive myeloid cells obtained from patients with chronic myelogenous leukemia. In the initial phase of this protocol rGM-CSF was administered as the sole agent for up to 7 days to assess dose response and toxicity. All patients were studied during this phase of continuous infusion of GM-CSF and were not exposed to chemotherapy prior to or during the study period. Five patients (1 through 5) received GM-CSF at 32 μg/kg/d and four patients (6 through 9) received 64 μg/kg/d.

Immunofluorescence analysis. Peripheral blood was collected from patients and normal controls by venipuncture, collected into heparinized plastic syringes, and placed immediately at 4°C in polyurethane test tubes. Cell surface antigens were detected by indirect immunofluorescence using a method to stain leukocytes in whole (unseparated) blood (Coulter Immunology, Hialeah, FL). The whole blood technique was used to avoid any in vitro manipulation that would activate granulocytes. Briefly, 100 μL of whole blood was incubated at 4°C with monoclonal antibody for ten minutes; this was followed by two washes in phosphate-buffered saline (PBS). The cells were then further incubated with fluoresceinated antimouse Ig (Coulter Immunology) for five minutes at 4°C followed by two additional washes in PBS. Lysis of erythrocytes was then accomplished using the Coulter lysis reagent. The leukocytes were then fixed by using a formaldehyde-methanol-based fixative and subsequently analyzed by flow cytometry in a Coulter EPICS-C flow cytometer. Granulocyte “windows” were set on the basis of 90° light scatter, and only this fraction was analyzed for surface fluorescence. The cells in this window were shown to be greater than 95% granulocytes by sorting into collection tubes and examining cytocentrifuge smears following Wright-Giemsa staining. Also, the cells in the granulocyte window were shown to lack reactivity (less than 5%) with monoclonal antibodies identifying other lineages. These included anti-Mo2 (CD14, monocytes), anti-T3 (CD3, T lymphocytes), and anti-B1 (CD20, B lymphocytes). CD11b was detected with monoclonal antibody 9044 and CD11a detected with monoclonal antibody 2F12 (the latter kindly provided by Dr Jerome Ritz, Dana-Farber Cancer Institute, Boston). CD11c was detected by monoclonal anti-Leu-M5 (Becton Dickinson, Mountain View, CA). Other antibodies tested included anti-B7.3.19 anti-MY1,20 anti-MY4,21 anti-MY7,21 anti-MY8,21 anti-MY9,22 and anti-BOH.5.23 All antibodies were used at saturating concentrations and were obtained from the original investigators. In one experiment the CD11b-positive granulocytes were separated into high- and low-density CD11b fractions by fluorescence-activated cell sorting. Cytospin preparations of the fractions were subsequently stained (Wright Giemsa), and 200 cell differential counts were performed by three individuals.

Estimation of change in surface antigen density. Fluorescence intensity measured by flow cytometry is proportional to the number of fluorescein molecules bound per cell. When a linear amplifier is used, fluorescence intensity is linearly related to the channel number on the flow cytometer. Estimation of the amount of increase in surface antigen expression was performed by comparing the peak fluorescence intensity of known monoclonal antibodies for which the exact number of binding sites per cell had been previously determined. For this purpose, purified T cells were obtained by standard sheep cell rosetting techniques. A standard curve relating logarithmic to linear fluorescence was constructed by using anti-T4 (IgG2) and anti-T8 (IgG2) monoclonal antibodies. For anti-T4 and anti-T8, relative linear fluorescence intensities of 3.0 and 6.0 (arbitrary units), respectively, were assigned on the basis of the known 1:2 relationship of the number of binding sites per cell. CD11b peak channel fluorescence measurements were then converted from a logarithmic scale to a linear scale, thereby allowing accurate assessment of relative changes in surface antigen density.

In vitro studies. The in vitro effects of rGM-CSF on human granulocytes were examined by adding rGM-CSF (obtained as media conditioned by Chinese hamster ovary (CHO) cells transfected with a full-length cDNA clone of the human GM-CSF gene, kindly provided by Drs Steven Clark and Gordon Wong, Genetics Institute, Cambridge, MA) to whole blood aliquots at a dilution of 1:500. Peripherial blood was incubated with GM-CSF for 20 minutes at 37°C. Controls included peripheral blood incubated with “mock” CSF (media harvested from CHO cells transfected with the same vector lacking the GM-CSF CDNA) or with media alone for 20 minutes at 37°C. Cell surface staining with monoclonal antibodies was then performed as described earlier.

RESULTS

Effects of GM-CSF on normal granulocyte CD11a and CD11b expression in vitro. Whole blood from normal individuals was incubated at 37°C for 20 minutes with rGM-CSF, mock CSF, and media alone. The surface expression of CD11b on mature granulocytes was analyzed by the whole blood lysis technique and increased 2.3 ± 0.17-fold after exposure to GM-CSF. This was significantly different when compared with mature granulocytes incubated with mock CSF (1.3 ± 0.15, P = .013) or media alone (1.3 ± 0.12, P = .013). In contrast, there was no significant increase in the surface expression of CD11a in either CM-CSF, mock CSF–treated whole blood, or the whole blood exposed to incubation alone. Importantly, there was no significant change in overall cell size (estimated by the light scatter profile) after exposure to GM-CSF, mock CSF, or media alone, which showed that the increase in CD11b expression was not due to an overall increase in cell surface area.

Effects of GM-CSF in vivo. The surface expression of CD11b on granulocytes pre–GM-CSF and 12 hours post–GM-CSF in nine patients is depicted in Table 1. There was a significant increase in CD11b-positive granulocytes (53% ± 9% v 89% ± 5%, P = .0001) as well as surface expression of CD11b (mean fold increase, 2.05 ± 0.22; P = .002) after 12 hours of treatment with GM-CSF. There was no change in overall cell size (estimated by light scatter). The surface expression of CD11a or CD11c on mature granulocytes after 12 hours of treatment with GM-CSF did not significantly change, nor did the percentage of CD11a- or CD11c-positive granulocytes. In one patient, the surface expression of B7.3.1, MY1, MY4, MY7, MY8, MY9, and 8OH.5 did not change significantly after 12 hours of treatment with GM-CSF (data not shown). There did not appear to be a significant dose-response effect with regard to fold increase in CD11b surface expression when comparing the five patients treated at 32 μg/kg/d (1.76 ± 0.13) v the four patients treated at 64 μg/kg/d (2.42 ± 0.42, P = .14). Since the GM-CSF was
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In one patient (no. 3) only CD11b surface expression was monitored. In the other patient, a lesser decrease in CD11b expression occurred (40% reduction by day 5). In the three patients showing a decrease in CD11b expression, the effect on CD11b surface expression to the pretreatment baseline by 36 hours after initiation of GM-CSF treatment. In the other patient, a lesser decrease in surface expression was observed (40% reduction by day 5).

In the three patients who showed either constant or increased surface expression of CD11b following initiation of GM-CSF treatment, the CD11b fluorescence histogram broadened on subsequent days, thus suggesting the presence of several populations of cells. To investigate the nature of these populations, the high-density CD11b-positive fraction (upper 25th percentile) was separated from the low-density CD11b-positive fraction using density gradient centrifugation.

Table 1. CD11b Surface Expression on Granulocytes Pre–GM-CSF and 12 Hours Post–GM-CSF Treatment

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Relative Fluorescence Intensity*</th>
<th>CD11b-Positive Granulocytes (%)†</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.54</td>
<td>1.00</td>
<td>1.95</td>
</tr>
<tr>
<td>2</td>
<td>0.50</td>
<td>0.76</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>0.50</td>
<td>0.76</td>
<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>0.76</td>
<td>1.65</td>
<td>2.2</td>
</tr>
<tr>
<td>5</td>
<td>0.60</td>
<td>1.04</td>
<td>1.7</td>
</tr>
<tr>
<td>6</td>
<td>0.90</td>
<td>2.00</td>
<td>2.2</td>
</tr>
<tr>
<td>7</td>
<td>0.50</td>
<td>0.82</td>
<td>1.6</td>
</tr>
<tr>
<td>8</td>
<td>0.84</td>
<td>1.90</td>
<td>2.3</td>
</tr>
<tr>
<td>9</td>
<td>0.63</td>
<td>2.30</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Mean ± SEM 0.64 ± 0.05 1.36 ± 0.20 2.05 ± 0.22 53 ± 9 89 ± 5

*Values represent relative linear fluorescence intensity obtained from the peak channel of fluorescence as described in the Materials and Methods.†Percent positive granulocytes determined by flow cytometry using indirect immunofluorescence. Background fluorescence (isotype-identical control antibody) was subtracted.‡Patients 4 and 5 had the surface expression of CD11b measured after 24 hours of GM-CSF treatment. From www.bloodjournal.org by guest on August 16, 2017. For personal use only.
Fig 2. In vivo effect of continuous infusion GM-CSF on CD11b, CD11a, and CD11c surface expression on granulocytes. Surface antigen density is depicted on the horizontal axis. At 15 minutes, the total WBC count was $0.7 \times 10^9/\mu L$ with 20% granulocytes, which made analysis of large numbers of granulocytes difficult. This accounts for the low amplitude of the histogram at 15 minutes.

fraction (lower 25th percentile) by fluorescence-activated cell sorting in patient no. 7 on day 5. The one parameter analysis of the presort cell population analyzed is shown in Fig 4A along with the windows utilized for the cell sort. Figure 4B shows a comparison of the histograms of the two populations postsort with clear separation of two populations of cells having differing relative fluorescence intensities. Cytospin preparations of these two populations revealed a significantly higher proportion of mature granulocytes in the high-density CD11b-positive fraction compared with the low-density CD11b-positive fraction (Table 2). Conversely, a significantly higher proportion of more immature myeloid cells (metamyelocytes and band forms) was seen in the low-density CD11b-positive fraction compared with the high-density CD11b-positive fraction. These data suggest that the decrease in in vivo surface expression of CD11b over time (despite continued infusion of GM-CSF) is partly due to increased numbers of more immature myeloid cells possessing a lower density of CD11b. As shown in Figs 3B, 3D, and 3F, the number of immature myeloid cells circulating in the peripheral blood of these patients treated with 5 days of GM-CSF increases dramatically, ultimately accounting for 35% to 50% of the total number of circulating leukocytes (compared with 0% to 2% prior to treatment with GM-CSF).

The surface expression of CD11a on mature granulocytes of three patients treated with up to five days of GM-CSF was examined. There was no significant change in the surface expression of CD11a on mature granulocytes after 12 hours of treatment in four patients analyzed. In one of the three patients studied on subsequent days, there was a significant increase in the surface expression of CD11a on mature granulocytes that occurred on day 3 of treatment. In the two other patients, no major increase in CD11a surface expression was seen.

**DISCUSSION**

CD11b (Mol) is a unique cell surface protein expressed by granulocytes and monocytes that appears to be critically involved in phagocyte adhesion-dependent functions, including granulocyte aggregation following activation, adherence to endothelial cells, and chemotaxis. CD11b is also the receptor for iC3b, an opsonic fragment of the third component of complement that can be fixed to the surface of microorganisms during complement activation. CD11b has also been shown to act synergistically with receptors for the Fc portion of IgG to enhance phagocytosis of serum-opsonized microorganisms. Deficiency of CD11b is a rare autosomal recessive disorder affecting granulocyte adherence-related functions and is characterized clinically by depressed inflammatory responses and recurrent infections.

Given the critical role that CD11b plays in mediating granulocyte adherence-related functions, the factors that regulate CD11b expression may be important in modulating granulocyte function. Peripheral blood granulocytes as well...
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One such syndrome is the granulocytopenia observed in enhanced granulocyte aggregation, possibly mediated by could be inhibited by a monoclonal antibody directed against CD11b that of phane dialysis membranes. In these patients maximal on new copper- increased expression of CD11b, is thought to be pathogenic. Several clinical syndromes have been described in which myelocytes have been shown to possess intracellular pools of CD11b that can be rapidly mobilized to the cell surface after granulocyte activation. Stimuli that are known to increase the surface expression of CD11b in vitro include f-Met-Leu-Phe, phorbol ester, complement C5a, calcium ionophore A23187, tumor necrosis factor (TNF), and GM-CSF. With regard to GM-CSF, Arnaout et al have shown that in vitro exposure of granulocytes to GM-CSF will rapidly increase the surface expression of CD11b with no immediate effect on the expression of CD11a. The magnitude of the increase is approximately 2.8-fold and occurs within 15 minutes. The increased expression of CD11b was associated with enhanced aggregation of granulocytes that could be inhibited by a monoclonal antibody directed against CD11b.

Several clinical syndromes have been described in which enhanced granulocyte aggregation, possibly mediated by increased expression of CD11b, is thought to be pathogenic. One such syndrome is the granulocytopenia observed in hemodialysis patients undergoing dialysis on new cuprophone dialysis membranes. In these patients maximal decreases in granulocyte counts occur within minutes and correlate with the peak increase in the surface expression of CD11b on granulocytes. The increase in CD11b surface expression on granulocytes is thought to be mediated by activated complement components produced by the cuprophone membrane. Leukoaggregation and pulmonary sequestration of granulocyte microaggregates is believed to be the mechanism of the granulocytopenia. Pulmonary dysfunction in the form of hypoxemia and hypocapnia and a decrease in diffusing capacity occurs in these patients. Complement- activated granulocytes have also been shown to have enhanced adhesiveness to umbilical vein endothelial cells. In addition, a recent study has shown that increased CD11b expression induced by TNF leads to enhanced binding of granulocytes to umbilical vein endothelium. In animal models of neutrophil-induced pulmonary injury, the damage to pulmonary endothelium and epithelium that is produced by activated granulocytes can be reduced by treatment of activated granulocytes with monoclonal antibodies directed against CD11b.

On the basis of these observations, it is likely that enhanced expression of the CD11b molecule in vivo may have potentially important clinical consequences. Therefore, in view of the ability of GM-CSF to stimulate CD11b expression in vitro, we evaluated the effects of this growth factor on granulocyte CD11b expression in patients receiving continuous-infusion GM-CSF as part of a phase I clinical trial. We now report that the continuous infusion of GM-CSF will significantly increase the surface expression of CD11b on mature granulocytes in vivo. The increase in CD11b was seen within 30 minutes of administration of GM-CSF in two of three patients studied and was present 12 to 24 hours later in all nine patients. On subsequent days, the CD11b level decreased in some patients, and this phenomenon was at least partially attributable to the dramatic rise in the immature myeloid forms in the peripheral blood during treatment with GM-CSF. These immature myeloid forms were shown to have a lower surface density of CD11b compared with mature granulocytes. Previous studies have also suggested that CD11b is not present on myeloid precursor cells but is acquired with differentiation.

Transient leukopenia induced by GM-CSF has been previously reported. Using radionuclide-labeled leukocytes, it was shown that this occurs secondary to margination, primarily in the lung vasculature. No apparent toxicity was observed, although the authors raised the issue of potential toxicity in the setting of sepsis. Our data suggest that the transient leukopenia observed may be due to enhanced granulocyte aggregation and margination secondary to the...
rapid increase in CD11b surface expression induced by GM-CSF. This is similar to the mechanism previously documented in the granulocytopenia of hemodialysis. With recovery of the WBC count, CD11b surface expression decreased from the peak value noted at 60 minutes following initiation of GM-CSF treatment. No immediate pulmonary toxicity of GM-CSF was apparent in our study. However, it is possible that given a second stimulus (ie, sepsis), activated granulocytes that have margined within the pulmonary microvasculature could cause endothelial damage. It is of potential interest that two of four patients receiving 64 μg/kg/d of GM-CSF in our study developed edema, granulocytes from decreased in the granulocytopenia of hemodialysis. With documented GM-CSF. This is similar to the mechanism previously in rapid increase.

Human GM-CSF can now be produced in large quantities, and future trials in humans will undoubtedly focus on the effect of this biologic agent on chemotherapy-induced myelosuppression, bone marrow transplantation, and neutrophil and monocyte functions in vivo. The toxicity of GM-CSF in humans has yet to be defined fully. Although the enhancement of adhesion proteins on the surface of mature granulocytes by GM-CSF in vivo may serve to retain granulocytes at sites of inflammation and improve their cytotoxic function, granulocyte activation could potentially have deleterious effects as well. Enhanced expression of CD11b in vitro correlates with enhanced aggregation of granulocytes and enhanced adherence to endothelial cells. GM-CSF both in vitro and in vivo in both primates and humans increases the production of superoxide anion by granulocytes, which is believed to mediate endothelial cell damage by complement-stimulated granulocytes. Stimuli such as endotoxin introduced into a patient whose granulocytes have been primed with GM-CSF may lead to an exuberant inflammatory response and result in enhanced endothelial cell damage. These theoretical concerns regarding GM-CSF should not impede clinical trials designed to assess the efficacy of GM-CSF. However, our results should serve to heighten awareness of potential toxic effects on the host.

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Granulocyte-macrophage colony-stimulating factor induces the expression of the CD11b surface adhesion molecule on human granulocytes in vivo

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