Prolongation of Survival of Human Polymorphonuclear Neutrophils by Granulocyte-Macrophage Colony-Stimulating Factor Is Caused By Inhibition of Programmed Cell Death

By Marion A. Brach, Sven deVos, Hans-Jürgen Gruss, and Friedhelm Herrmann

In the absence of appropriate stimuli, polymorphonuclear neutrophils (PMN) undergo programmed cell death (PCD), also termed apoptosis. We show that granulocyte-macrophage colony-stimulating factor (GM-CSF), but not the chemotactic factors formyl-methionyl-leucyl-phenylalanine (FMLP), recombinant human (rh) C5a, transforming growth factor (TGF)-β, and interleukin-8 (IL-8), or other cytokines including IL-3, IL-4, IL-6, and G-CSF, maintains viability of PMN in culture by preventing these cells from undergoing PCD. Prevention from PCD by GM-CSF was associated with induction of RNA and protein synthesis in PMN. Inhibition of RNA and protein synthesis by actinomycin-D and cycloheximide impeded the protection of apoptosis by GM-CSF. Similarly, neutralization of GM-CSF biologic activity by a specific antiserum abrogated GM-CSF-mediated inhibition of PCD.

MATERIALS AND METHODS

Preparation of PMN. Heparinized venous blood was obtained from six age- and sex-matched consenting healthy volunteers. Neutrophils were isolated by dextran sedimentation and centrifugation on a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) cushion as previously described. Cells were then subjected to another density cut using centrifugation over a Percoll (Pharmacia) gradient to isolate PMN from contaminating eosinophils and basophils. PMN (>98% pure by morphology) were then resuspended at 1 x 10⁶ cells/mL in RPMI 1640 medium (Flow Laboratories Ltd, Irvine, Scotland) supplemented with 100 U/mL penicillin, 100 U/mL streptomycin, 2 mmol/L L-glutamine (Serva, Heidelberg, Germany), and 10% autologous fresh serum (standard culture medium; SCM).

Test factors. Recombinant human cytokines including IL-3 (kindly provided by S. Gillis, Immunix, Seattle, WA), IL-4 (kindly provided by P. Trotta, Schering Plough, Bloomfield, NJ), IL-6 (kindly provided by T. Hirano, Osaka University, Osaka, Japan), IL-8 (Genzyme Corp, Boston, MA), GM-CSF (kindly provided by S. Gillis, G-CSF (kindly provided by L. Souza, Amgen, Thousand Oaks, CA), TGF-β (R&D Systems, Minneapolis, MN), as well as FMLP (Peninsula Laboratories, Belmont, CA), rh C5a, actinomycin-D (Act-D), or cycloheximide (CHX) (Sigma, Munich, Germany) were diluted in SCM in concentrations as indicated (saturation binding conditions). Lipopolysaccharide (LPS) levels in media and test factors were determined using the Limulus lysate assay and were found to be less than 5 pg/mL, a concentration insufficient to modulate PCD of PMN in pilot experiments.

Culture of PMN. PMN were incubated for a period of up to 216 hours in tissue culture tubes (17 x 100 mm; Falcon, Becton Dickinson, Heidelberg, Germany) at 37°C in a humidified atmosphere of 5% CO₂ in air. The cultures were performed in the presence or absence of the above test factors. In selected experiments GM-CSF was preincubated for 16 hours at 4°C with a specific neutralizing sheep anti-GM-CSF antiserum (kindly provided by G. Wong, Genetics Institute, Cambridge, MA) before incorporation into experimental cultures. Anti-GM-CSF antiserum was used in concentrations sufficient to neutralize 5 mmol/L rhGM-CSF (assessed in pilot experiments). At various times, viable cells were counted by the eosin dye exclusion test.

RNA and protein synthesis by PMN. PMN (1 x 10⁶/mL) resuspended in SCM were incubated for up to 48 hours with rhGM-CSF (1 mmol/L) in the presence or absence of Act-D (2 μg/mL) or CHX (10 μg/mL) at 37°C in 5% CO₂ in air. During the last 6 hours cultures received 2.8 x 10⁶ kBq/mL [³H]-uridine (specific activity 1.37 G Bq/mmol; Amersham Buchler, Braunschweig, Germany) or 1 x 10⁴ kBq/mL [³H]-leucine (specific activity 2.74 TBq/mmol; Amersham Buchler). Incorporation of [³H]-uridine or [³H]-leucine was measured by liquid scintillation counting (Packard, Meriden, CT). Results are expressed as cpm x 10⁻³.
DNA fragmentation analysis. DNA fragmentation in PMN was analyzed as previously described. In brief, $5 \times 10^6$ cells were incubated at 37°C for 4 hours in 500 μL of lysis buffer (200 mmol Tris, pH 8.5, 100 mmol/L EDTA, 50 μg/mL proteinase K, 1% sodium dodecyl sulfate). The DNA was phenol-chloroform extracted and then dialyzed for 12 hours against 10 mmol/L Tris (pH 7.5), 1 mmol/L EDTA. After dialysis the DNA solution was treated with RNase A, and further incubated with 150 μg/mL of proteinase K for 5 hours at 37°C. The DNA was extracted with phenol and chloroform and precipitated with ethanol. Each sample was electrophoresed on agarose gels (1%) with TBE buffer (89 mmol/L Tris, 89 mmol/L borate, 2 mmol/L EDTA) containing 0.5 μg/mL ethidium bromide.

RESULTS

Effect of chemotactic factors and cytokines on PMN survival. It has been previously shown that peripheral blood-derived PMN do not survive more than 72 to 96 hours in vitro without the addition of survival-promoting cytokines. We have analyzed a variety of chemotactic factors and cytokines participating in the inflammatory process for their capacity to maintain PMN survival in vitro. FMLP, rhC5a, TGF-β, IL-8, IL-3, IL-4, IL-6, and G-CSF caused no change in the percentage of neutrophil survival in culture. However, when the culture medium was supplemented with rhGM-CSF, PMN survived at least 216 hours (Table 1). Sufficient levels of GM-CSF were necessary to maintain viability of PMN in cultures because removal of exogenous GM-CSF by repeated washing procedures 12 hours after initiation of culture caused progressive loss of cell viability (Fig 1). The capacity of GM-CSF to prevent death of PMN was specific in that it could be reversed by a neutralizing anti-GM-CSF antiserum (Fig 2).

GM-CSF inhibits DNA fragmentation of PMN. When PMM were cultured in vitro, most of them exhibited features characteristic of apoptotic cells before dying, ie, nuclear condensation, pyknosis, and cytoplasmic vacuolation. Therefore, we asked whether the dead of PMN accompanies DNA fragmentation, and if so, whether GM-CSF inhibits this process. To that end DNA fragmentation analysis of PMN on agarose gel electrophoresis was performed. As shown in Fig 3, DNA fragmentation was readily detectable in PMN cultured for 24 hours. FMLP, rhC5a, TGF-β, IL-8, IL-3, IL-4, IL-6, and G-CSF were incapable of rescuing PMN from PCD (ie, from DNA fragmentation). However, DNA fragmentation was prevented in the presence of GM-CSF in culture. DNA fragmentation in PMN became first detectable 6 to 8 hours after initiation of cultures, which preceded the morphologically detectable death of PMN by 24 to 36 hours (not shown).

De novo RNA and protein synthesis is required to protect PMN against DNA fragmentation. We next examined whether the GM-CSF-induced prolongation of survival of PMN required the synthesis of new RNA and protein. Figure 4 shows that neutrophil survival was not supported by GM-CSF in the presence of inhibition of RNA synthesis by Act-D or inhibition of protein synthesis by CHX. This observation indicated that the GM-CSF-induced survival of PMN required de novo RNA and protein synthesis. Complementarily, we determined whether GM-CSF induces new RNA and protein synthesis in PMN. As shown in Table

Table 1. In Vivo Survival of Human Peripheral Blood-Derived PMN in the Presence of FMLP, rhC5a, rhTGF-β, rhIL-8, rhGM-CSF, rhIL-3, rhIL-4, rhIL-6, and rhG-CSF

<table>
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<tr>
<th>Time in Culture (h):</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
<th>144</th>
<th>168</th>
<th>192</th>
<th>216</th>
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<tbody>
<tr>
<td>SCM</td>
<td>100</td>
<td>68 ± 4</td>
<td>18 ± 3</td>
<td>7 ± 1</td>
<td>0</td>
<td>0</td>
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<tr>
<td>FMLP (5 nmol/L)</td>
<td>100</td>
<td>70 ± 9</td>
<td>18 ± 5</td>
<td>9 ± 6</td>
<td>2 ± 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>rhC5a (1 nmol/L)</td>
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<td>62 ± 6</td>
<td>8 ± 3</td>
<td>2 ± 2</td>
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<td>rhTGF-β (100 pmol/L)</td>
<td>100</td>
<td>60 ± 4</td>
<td>8 ± 5</td>
<td>4 ± 2</td>
<td>1 ± 1</td>
<td>0</td>
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<td>rhIL-8 (1 nmol/L)</td>
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<td>89 ± 9</td>
<td>16 ± 4</td>
<td>7 ± 3</td>
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<td>100</td>
<td>80 ± 9</td>
<td>81 ± 9</td>
<td>87 ± 11</td>
<td>92 ± 9</td>
<td>96 ± 3</td>
<td>97 ± 2</td>
<td>98 ± 2</td>
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<td>rhIL-3 (1 nmol/L)</td>
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<td>71 ± 10</td>
<td>19 ± 7</td>
<td>8 ± 4</td>
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<td>rhIL-4 (1 nmol/L)</td>
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<td>70 ± 8</td>
<td>19 ± 8</td>
<td>10 ± 5</td>
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<td>rhIL-6 (1 nmol/L)</td>
<td>100</td>
<td>70 ± 8</td>
<td>18 ± 8</td>
<td>6 ± 3</td>
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<td>0</td>
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<td>rhG-CSF (1 nmol/L)</td>
<td>100</td>
<td>74 ± 6</td>
<td>24 ± 6</td>
<td>13 ± 4</td>
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<td>2 ± 1</td>
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</table>

*Neutrophils were cultured at $1 \times 10^6$ cells/mL in the presence or absence of the indicated test factors for a period of up to 216 hours. At various times as indicated viable cells were counted by the eosin dye exclusion test. Data from five neutrophil donors are shown and are expressed as means ± SD.
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Effect of inhibition of RNA synthesis (Act-D treatment) and inhibition of protein synthesis (CHX treatment) on prolongation of PMN in vitro survival by GM-CSF. PMN (1 x 10^6/mL) were cultured for a period of 96 hours in the absence (SCM) or presence of rhGM-CSF (1 nmol/L) with or without Act-D (2 µg/mL) or CHX (10 µg/mL). Representative data from a single neutrophil donor are shown and are expressed as means of triplicated samples that varied by less than 9% of the mean. Analysis with neutrophils from three other donors gave comparable results. (---), SCM; (○), GM-CSF; (□), GM-CSF + Act-D; (■), GM-CSF + CHX.

Fig. 3. Analysis in agarose gels of DNA isolated from human peripheral blood derived PMN cultured in the presence or absence of FMLP (5 nmol/L), rhC5a (1 nmol/L), rhTGF-β (100 pmol/L), rhIL-8 (1 nmol/L), rhGM-CSF (1 nmol/L), rhIL-3 (1 nmol/L), rhIL-4 (1 nmol/L), rhIL-6 (1 nmol/L), rhG-CSF (1 nmol/L) for a period of 24 hours. Representative data from a single neutrophil donor are shown. Analysis with neutrophils from two other donors gave comparable results.

Fig. 4. Effect of antibody neutralization on GM-CSF-induced inhibition of PMN PCD. PMN (1 x 10^6/mL) were cultured for a period of 72 hours with SCM only or a medium containing rhGM-CSF that had been preincubated without anti-GM-CSF antiserum (▲) or with anti-GM-CSF antiserum (□). Representative data from a single neutrophil donor are shown and are expressed as means of duplicate samples that varied by less than 7.5% of the mean. Analysis with neutrophils from two other donors gave comparable results. (■), No antibody; (○), anti–GM-CSF.

Effect of Act-D, CHX, and neutralizing antiserum to GM-CSF on GM-CSF-mediated protection of DNA fragmentation. As shown in Fig 5, the capacity of GM-CSF to prevent DNA fragmentation of PMN was reversed by the presence of Act-D or CHX in culture. Neither Act-D or CHX alone prevented DNA fragmentation. Similarly, DNA fragmentation also occurred in PMN incubated in GM-CSF that was pretreated with neutralizing concentrations of an antiserum to rhGM-CSF.

DISCUSSION

PCD represents a physiologic mechanism for deleting cells from certain tissues without inducing inflammation and subsequent damage to contiguous cells. Previous studies by others have indicated that the survival of a variety of different cell species requires the presence of specific cytokines to maintain viability. When deprived of these cytokines, the cells undergo PCD. For instance, survival of erythropoietic progenitor cells is dependent on the pres-
GM-CSF PREVENTS PCD OF NEUTROPHILS

Table 2. RNA and Protein Synthesis in Human Peripheral Blood-Derived PMN

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$^{3}$H-Uridine Uptake (cpm x 10$^{-3}$)</th>
<th>$^{3}$H-Leucine Uptake (cpm x 10$^{-3}$)</th>
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<tr>
<td>SCM</td>
<td>0.71 ± 0.09</td>
<td>1.09 ± 0.12</td>
</tr>
<tr>
<td>rhGM-CSF</td>
<td>2.1 ± 0.23</td>
<td>4.97 ± 0.45</td>
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<tr>
<td>rhGM-CSF + CHX</td>
<td>ND</td>
<td>0.83 ± 0.07</td>
</tr>
<tr>
<td>rhGM-CSF + Act-D</td>
<td>0.65 ± 0.04</td>
<td>ND</td>
</tr>
<tr>
<td>rhG-CSF</td>
<td>0.83 ± 0.09</td>
<td>1.16 ± 0.13</td>
</tr>
<tr>
<td>rhL-3</td>
<td>0.71 ± 0.08</td>
<td>1.05 ± 0.15</td>
</tr>
<tr>
<td>rhC5a</td>
<td>0.67 ± 0.05</td>
<td>0.97 ± 0.12</td>
</tr>
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</table>

Neutrophils (1 x 10$^6$ cells/mL) were cultured in the presence or absence of rhGM-CSF, rhG-CSF, rhL-3, rhC5a (1 nmol/L each), Act-D (2 μg/mL), CHX (10 μg/mL) as described in Materials and Methods. RNA synthesis ($^{3}$H-uridine uptake) was measured after 24 hours of culture, protein synthesis ($^{3}$H-leucine uptake) after 48 hours. Data from 4 neutrophil donors are shown and are expressed as means ± SD. Concomitantly, DNA synthesis ($^{3}$H-thymidine uptake) was measured in all cases but no changes were detected.

Abbreviation: ND, not done.

ence of erythropoietin, while IL-6 inhibits apoptosis of early myelopoietic cells. Withdrawal of IL-2 causes PCD in dependent T lymphocytes. Monocytes are dependent on IL-1 and tumor necrosis factor-α for survival. Eosinophils require IL-5, and in neuronal cells nerve growth factor prevents DNA breakdown and PCD. In this report we have shown that GM-CSF can maintain neutrophil viability by preventing these cells from undergoing PCD. Other cytokines, including IL-3, IL-4, IL-6, and G-CSF, have no effect on this process. Moreover, factors known to attract PMN to inflammatory sites, namely FMLP, C-fragment C5a, TGF-β, and IL-8, also have no significant effect on PMN survival. Therefore, these results suggest that PMN recruited into inflammatory lesions by chemotactic factors will nonetheless die by PCD unless activated by certain “trophic hormones” such as GM-CSF. GM-CSF is mainly produced by activated T lymphocytes and monocytes, thus suggesting that a paracrine interaction of these cells with PMN regulates PCD of PMN. A local decrease of GM-CSF production, as it would be expected when inflammation wanes, could lead to the onset of PMN PCD and thus would control the number of PMN in an inflammatory lesion.

Cleavage of chromosomal DNA into oligonucleosome-sized fragments is a hallmark feature of PCD. We show that GM-CSF which maintained survival of PMN also prevented DNA fragmentation. Both promotion of PMN survival and prevention of fragmentation of their DNA by GM-CSF was not maintained in the presence of inhibition of RNA and protein synthesis by Act-D and CHX, suggesting that some proteins inhibiting endonuclease activity might be produced by PMN in response to stimulation by GM-CSF. Indeed, upon exposure to GM-CSF in PMN, significant RNA and protein synthesis but no DNA synthesis was observed. However, RNA and protein synthesis did not take place when Act-D and CHX were added to GM-CSF-stimulated PMN cultures. In addition, no RNA and protein synthesis was observed in PMN incubated with IL-3, G-CSF, or rhC5a. In conclusion, our findings suggest that the prolonged survival of PMN by GM-CSF is associated with newly synthesized proteins that prevent PCD.

Until now no particular endonuclease involved in PCD has been identified. However, recently certain nuclear proteins that increase during induction of PCD have been
defined as histone proteins.\textsuperscript{19} Moreover, overexpression of the proto-oncogene \textit{bcl-2} has been associated with prolongation of cell survival\textsuperscript{20} and the product of the mutant tumor suppressor gene \textit{p53} has been shown to block the death of apoptotic cells.\textsuperscript{8} Therefore, it will be interesting to analyze in future studies whether alteration of \textit{bcl-2} or \textit{p53} expression or induction of histone-type nucleases will result from treatment of PMN with GM-CSF.

**REFERENCES**

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