Further Study of Internal Autocrine Regulation of Multipotent Hematopoietic Cells

By Nancy Pech, Olivier Hermine, and Eugene Goldwasser

We have extended the study of the effects of antisense oligodeoxynucleotides on hematopoietic colony formation to include the effects of antisense to granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and macrophage colony-stimulating factor (M-CSF) on bone marrow cultures. GM-CSF antisense and GM-CSF receptor antisense cause an increase in mixed erythroid:nonerythroid colonies and a decrease in mixed nonerythroid colonies, which is an effect opposite to that described previously for erythropoietin (Epo) and Epo receptor antisense. The effect of GM-CSF antisense oligomer is not abrogated by the presence of the ligand in the culture. Antisense oligomers to G-CSF and M-CSF have no effect when Epo and GM-CSF antisense oligomers are added simultaneously, the effects seem to be independent, with the GM-CSF antisense predominating. These data support the hypothesis of internal autocrine regulation of multipotent hematopoietic precursor cells, and extend the concept to myeloid as well as erythroid differentiation.

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MATERIALS AND METHODS

Oligodeoxynucleotides preparation. Oligomers were synthesized on an Applied Biosystems 380B DNA synthesizer (Applied Biosystems, Inc, Foster City, CA).

The sequences used were from the mouse genes for GM-CSF, G-CSF, and M-CSF. Each exon I sense sequence starts three bases from the translation initiation site. The GM-CSF sense sequence is 5'TGGCTGCAGAATTTACCT3' and the complementary antisense sequence is 5'AGTAAATTTCTGCAGCC3'. The G-CSF sense sequence is 5'GCTCAACATTGTGCCC3'. The complementary antisense is 5'CTGGGGAGAAGTGGACG3'. M-CSF sense has the sequence 5'AGCTGCCCCGATGACCGC3', and the antisense sequence is 5'GGCTCATACGCGGACG3'. GM-CSF-R sense sequence is 5'ATAGGGTCATACGCCC3', and the antisense sequence is 5'GGGATGTTGACCTA3'.

Cell culture. Marrow cells from 6- to 8-week-old B6D2F1/j6 female mice (Jackson Laboratories, Bar Harbor, ME) were flushed into α medium and resuspended at 10^7/mL in 70% α medium (Sigma Chemical Co, St Louis, MO) and 30% fetal calf serum (FCS) (GIBCO-BRL, Grand Island, NY) that had been heated to 66°C for 45 minutes. Cell suspensions (2 mL) containing 3 U/mL of murine recombinant interleukin-3 (IL-3) (courtesy of Dr Phillip B. Marines, Baxter Laboratories, Round Lake, IL) were incubated in 35-mm suspension culture dishes (Nunc, Inc, Naperville, IL). The appropriate oligodeoxynucleotides at 150 μg were added at zero time and 75 μg more added at 24 hours. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂, 95% air for 36 hours, after which 1 mL of α medium was added and the cells were harvested and centrifuged at 1,000 rpm (190g) for 10 minutes at 4°C. Cells were resuspended in 0.5 mL of α medium. The preincubated cells were plated at 10^5/mL in 0.8% methylcellulose (methylcel A4M premium; Dow Chemical, Midland, MI), 2-mercaptoethanol (0.1 mmol/L) (Eastman Kodak, Rochester, NY), sodium selenite (10 nmol/L) (ICN Pharmaceuticals, Plainview, NY), 2% bovine serum albumin (BSA) (Armour Pharmaceuticals, Kankakee, IL), 30% FCS (heated at 56°C for 30 minutes), 1 U/mL of Epo (recombinant human Epo, Amgen, Thousand Oaks, CA), and 50 U/mL of IL-3 (recombinant murine). The 1-mL cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂, 95% air for 7 or 11 days. The colonies were stained with benzidine according to Ogawa et al., and each of the four replicate dishes were scored for erythroid burst-forming units (BFU-E), mixed E:non-E, and mixed non-E colonies as previously described.

Statistical significance was calculated using Abstat software (Anderson-Bell Parker, CO). P values are from two-tailed t-tests. Be-
cause antisense oligomers (GM-CSF and GM-CSF-R) cause a significant decrease in total colony formation, t-tests were done using the percent of total values.

RESULTS

Incubation of mouse BM cells with GM-CSF antisense and sense oligonucleotides showed that the latter had essentially no effect on either the number or distribution of colonies at 7 or 11 days of culture. On the other hand, the antisense oligonucleotide caused a significant reduction (52%) of mixed non-E colonies, augmentation (41%) of mixed E:non-E colonies, and a decrease in total colonies (Table 1). At 7 days where erythroid bursts were found, the numbers were significantly decreased from the expected value because of the 36-hour preincubation and were too few to discern any effect on these purely erythroid colonies. The cultures incubated for 11 days showed similar effects of the antisense oligomers. These results were consistent over at least four experiments using the same protocol.

In contrast, G-CSF and M-CSF antisense oligonucleotides had no effect on colony formation at 7 or 11 days, as was also the case for the sense oligomers (Tables 2 and 3). Because these three experiments were done separately, and because it was important to show that the marrow cells could respond to the appropriate antisense oligomer, another experiment was done testing all six sense and antisense oligomers with the same pool of cells. The results at 7 days agreed with the individual experiments (Tables 1, 2, and 3), neither the sense nor antisense oligomers to GM-CSF or M-CSF had an effect, nor did the GM-CSF sense oligomer. The GM-CSF antisense oligomer, as previously, caused a significant increase in mixed E:non-E colonies, a significant decrease in mixed non-E colonies, and a significant decrease in total colonies (data not shown).

Because GM-CSF can be formed by some cells of the marrow and because the GM-CSF antisense might possibly affect non-E colony formation simply by inhibiting ligand formation by these cells, we tested the effects of the oligonucleotides in the presence of 100 U/mL of murine recombinant GM-CSF (rGM-CSF) (Immunex Corp, Seattle, WA) in the medium. The results show that neither the increase in mixed E:non-E colonies nor the decrease in mixed non-E colonies is altered by the presence of GM-CSF during the period when antisense oligonucleotides were present. This was true at both 7 and 11 days of culture (Tables 4 and 5).

The sequence of the murine GM-CSF receptor was recently published, and we could then test the effect of receptor sense and antisense oligomers on hematopoietic colony formation along with GM-CSF sense and antisense oligomers. The results (Table 6) with the receptor antisense agree with those using the ligand antisense. As expected, at both 7 and 11 days of culture, the receptor sense oligomer has no effect, whereas the receptor antisense 18 mer caused significant suppression of mixed non-E colony formation, a significant increase in mixed E:non-E colonies, and a significant decrease in total colonies.

It is important to note that even when there was a decrease in total colony formation caused by GM-CSF or GM-CSF-R antisense, the absolute number of mixed E:non-E colonies was increased (Tables 1, 4, 5, 6, and 7).

The evidence, to date, suggests that both Epo and GM-
CSF and their receptors may function in internal autocrine regulation of multipotent precursor cell proliferation and/ or differentiation, and that normal colony formation is decreased by the corresponding antisense oligomers. We tested the possibility that the actions of the two-ligand antisense oligomers are independent by adding both simultaneously and compared the effects with cultures in which the corresponding sense oligomers were added. The results (Table 7) are consistent with each antisense oligomer acting independently. For example, Epo antisense caused a 58% increase in mixed non-E colonies at 7 days as compared with the sense oligomer, whereas GM-CSF antisense caused a decrease of 62%. (We think it is appropriate to compare the effects of antisense oligomers with the corresponding sense oligomers rather than with the untreated controls because of the possible nonspecific effects of oligodeoxynucleotides on cell growth in cultures.) When both antisense oligomers were added, the increase in mixed non-E colonies caused by Epo antisense was essentially abrogated. Similarly, the epo antisense alone caused a 65% decrease in mixed E:non-E colony formation and GM-CSF antisense alone caused a small (19%) increase. When both were present, the decrease in mixed E:non-E colonies caused by Epo antisense was about one half that seen when only Epo antisense was present. With Epo antisense, the total number of colonies was slightly (11%) greater than that found with the sense oligomer, whereas for GM-CSF antisense total colony number was decreased by about 40%. When both antisense oligomers were present, the GM-CSF antisense effect predominated. Although total colony number was decreased when both antisense oligomers were present, the distribution of colonies was not affected by the antisense oligomers; 73% to 75% mixed non-E and 19% to 22% mixed E:non-E. This is in contrast to the effects of the GM-CSF antisense oligomer alone where there was a sharp decrease in the percentage of mixed non-E colonies and a sharp increase in the percentage of mixed E:non-E colonies.

**DISCUSSION**

There have been several reports of antisense effects on cytokine-responsive cells such as IL-1 β-responsive lymphokine-activated killer cells, IL-1α-responsive Th 2 cells, tumor necrosis factor alpha, (TNF-α)-responsive differentiating macrophages, M-CSF-responsive HL-60, FL-ras/myc cells, and FDC-P1 cells. In addition, IL-6-responsive cells were shown to be affected by its antisense oligomer. An effect of transforming growth factor beta (TGF-β) antisense on enhancement of colony formation from early hematopoietic cells was reported by Hatzfeld et al. An effect of Epo antisense on inhibition of human erythroleukemic cells was shown by Mitjavila et al. and of both Epo and Epo receptor antisense normal mouse marrow by our laboratory.

Our original observations on the effects of Epo and Epo receptor antisense oligomers on early progenitor cells of the marrow led us to the unorthodox and tentative conclusion that there is an internal autocrine mechanism that operates in the early stages of normal blood cell differentiation. This conclusion is strengthened by the data presented here, which show that, as anticipated from the model we pro-

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**Table 3. Lack of Effect of M-CSF Antisense Oligonucleotide on Colony Formation**

<table>
<thead>
<tr>
<th>Day 7</th>
<th>Mixed Non-E</th>
<th>Mixed E:Non-E</th>
<th>BFU-E</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>177 ± 12 (82 ± 1)</td>
<td>34 ± 4 (16 ± 1)</td>
<td>4 ± 1 (2 ± 1)</td>
<td>215 ± 14</td>
</tr>
<tr>
<td>M-CSF sense</td>
<td>155 ± 9 (83 ± 3)</td>
<td>29 ± 6 (16 ± 3)</td>
<td>3 ± 1 (2 ± 0)</td>
<td>187 ± 10</td>
</tr>
<tr>
<td>M-CSF antisense</td>
<td>159 ± 7 (82 ± 2)</td>
<td>32 ± 3 (16 ± 1)</td>
<td>4 ± 1 (2 ± 1)</td>
<td>186 ± 7</td>
</tr>
</tbody>
</table>

Day 11

| Control      | 229 ± 21 (89 ± 1)  | 27 ± 2 (11 ± 1)  | —     | 256 ± 23  |
| M-CSF sense  | 225 ± 22 (89 ± 2)  | 27 ± 2 (11 ± 2)  | —     | 253 ± 21  |
| M-CSF antisense | 212 ± 11 (90 ± 3) | 25 ± 4 (10 ± 3) | —     | 236 ± 16  |

Values are the means ±SD of four replicate cultures with percentage of total in parentheses.

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**Table 4. Effect of GM-CSF Antisense Oligonucleotide on 7-Day Colony Formation in the Presence of GM-CSF**

<table>
<thead>
<tr>
<th></th>
<th>Mixed Non-E</th>
<th>Mixed E:Non-E</th>
<th>BFU-E</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minus GM-CSF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>86 ± 3 (63 ± 1)</td>
<td>14 ± 2 (13 ± 2)</td>
<td>4 ± 1 (4 ± 1)</td>
<td>104 ± 5</td>
</tr>
<tr>
<td>GM-CSF sense</td>
<td>92 ± 11 (82 ± 2)</td>
<td>17 ± 4 (15 ± 3)</td>
<td>4 ± 2 (4 ± 2)</td>
<td>113 ± 13</td>
</tr>
<tr>
<td>GM-CSF antisense</td>
<td>38 ± 7 (52 ± 8)*</td>
<td>32 ± 5 (44 ± 7)*</td>
<td>3 ± 1 (5 ± 1)</td>
<td>73 ± 6*</td>
</tr>
</tbody>
</table>

Plus GM-CSF

<table>
<thead>
<tr>
<th></th>
<th>Mixed Non-E</th>
<th>Mixed E:Non-E</th>
<th>BFU-E</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>111 ± 10 (79 ± 5)</td>
<td>25 ± 6 (18 ± 5)</td>
<td>4 ± 1 (3 ± 1)</td>
<td>140 ± 7</td>
</tr>
<tr>
<td>GM-CSF sense</td>
<td>109 ± 13 (83 ± 5)</td>
<td>18 ± 6 (14 ± 5)</td>
<td>4 ± 1 (3 ± 1)</td>
<td>131 ± 11</td>
</tr>
<tr>
<td>GM-CSF antisense</td>
<td>54 ± 2 (59 ± 1)*</td>
<td>36 ± 1 (39 ± 2)*</td>
<td>2 ± 1 (3 ± 1)</td>
<td>92 ± 2*</td>
</tr>
</tbody>
</table>

100 U/mL of GM-CSF was present during the 36-hour preincubation. Values are the means ±SD of four replicate cultures with percentage of total in parentheses.

* Significance at P ≤ .025 when compared with sense control.
posed, an antisense oligomer directed against GM-CSF has an effect opposite to that found for Epo antisense. The suppression of mixed non-E colonies and augmentation of mixed E:non-E colonies suggest an internal autocrine role for GM-CSF and its receptor as well as for Epo and its receptor. Because the results are not affected by the addition of GM-CSF to the medium, we are justified in the assumption that the antisense effect is not simply the result of suppressed GM-CSF secretion by one subpopulation of marrow cells and acting on another in the more classic manner of external ligand:cell interaction. As we found earlier with Epo antisense, there was no discernible effect of the GM-CSF and GM-CSF-R antisense oligomers on the formation of the small number of erythroid bursts.

Our findings that both EpoR and GM-CSF-R antisense oligomers have effects similar to those of their corresponding ligands provide good evidence that the observed effects cannot be caused by the actions of accessory cells in the BM. Such cells might provide ligands but could not provide receptors.

In all of the experiments in which GM-CSF or its receptor antisense were present, there was a significant decrease in total colony formation that was not seen when Epo,1 M-CSF, or G-CSF antisense oligomers were used. This suppression of total colony formation occurred even when mixed E:non-E colony formation was increased. Because in control cultures the number of mixed non-E colonies ranges from three- to fivefold greater than the number of mixed E:non-E colonies, any significant suppression of granulocytic/monocytic differentiation by GM-CSF antisense is bound to cause a decrease in total number of colonies.

In contrast to our finding that G-CSF antisense does not have an effect on early hematopoietic cells, whereas GM-CSF antisense does, Segal et al22,23 reported that antisense for each of these cytokines can cause suppression of IL-1-induced expression of the corresponding growth factor.

The data suggesting independent action of Epo and GM-CSF antisense oligomer lend support to the idea that each of these ligands may interact with the corresponding receptor in an internal autocrine mode to regulate the proliferation and/or differentiation of multipotent precursor cells of the hematopoietic system. Other cytokine antisense oligomers are now being studied to determine how general this mechanism may be.

ACKNOWLEDGMENT

We are indebted to Drs Linda Park and Steven Gillis of Immunex Corporation for the gift of recombinant mouse GM-CSF.

Table 5. Effect of GM-CSF Antisense Oligonucleotide on 11-Day Colony Formation in the Presence of GM-CSF

<table>
<thead>
<tr>
<th></th>
<th>Mixed Non-E</th>
<th>Mixed E:Non-E</th>
<th>BFU-E</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minus GM-CSF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>87 ± 8 (81 ± 5)</td>
<td>21 ± 5 (19 ± 5)</td>
<td>—</td>
<td>108 ± 4</td>
</tr>
<tr>
<td>GM-CSF sense</td>
<td>87 ± 8 (60 ± 1)</td>
<td>20 ± 5 (20 ± 1)</td>
<td>—</td>
<td>109 ± 8</td>
</tr>
<tr>
<td>GM-CSF antisense</td>
<td>34 ± 5 (54 ± 6)*</td>
<td>29 ± 4 (47 ± 6)*</td>
<td>—</td>
<td>66 ± 2*</td>
</tr>
<tr>
<td>Plus GM-CSF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>81 ± 3 (75 ± 1)</td>
<td>28 ± 2 (25 ± 1)</td>
<td>—</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>GM-CSF sense</td>
<td>87 ± 4 (82 ± 2)</td>
<td>20 ± 2 (19 ± 2)</td>
<td>—</td>
<td>107 ± 4</td>
</tr>
<tr>
<td>GM-CSF antisense</td>
<td>53 ± 4 (60 ± 2)*</td>
<td>36 ± 1 (41 ± 2)*</td>
<td>—</td>
<td>89 ± 4*</td>
</tr>
</tbody>
</table>

100 U/mL GM-CSF was present during the 36-hour preincubation. Values are the means ±SD of four replicate cultures with percentage of total in parentheses.

* Significance at P ≤ .025 when compared with sense control.

Table 6. Effect of GM-CSF Receptor Antisense Oligonucleotide on Colony Formation

<table>
<thead>
<tr>
<th></th>
<th>Mixed Non-E</th>
<th>Mixed E:Non-E</th>
<th>BFU-E</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>166 ± 13 (87 ± 2)</td>
<td>20 ± 3 (10 ± 2)</td>
<td>4 ± 1 (3 ± 1)</td>
<td>190 ± 11</td>
</tr>
<tr>
<td>GM-CSF sense</td>
<td>155 ± 17 (84 ± 3)</td>
<td>24 ± 3 (13 ± 3)</td>
<td>5 ± 1 (3 ± 1)</td>
<td>184 ± 14</td>
</tr>
<tr>
<td>GM-CSF antisense</td>
<td>98 ± 13 (72 ± 9)*</td>
<td>36 ± 12 (26 ± 8)*</td>
<td>3 ± 2 (3 ± 1)</td>
<td>137 ± 9*</td>
</tr>
<tr>
<td>GM-CSF-R sense</td>
<td>151 ± 10 (88 ± 2)</td>
<td>20 ± 2 (11 ± 2)</td>
<td>6 ± 2 (3 ± 1)</td>
<td>175 ± 11</td>
</tr>
<tr>
<td>GM-CSF-R antisense</td>
<td>94 ± 8 (77 ± 1)*</td>
<td>25 ± 1 (21 ± 1)*</td>
<td>3 ± 2 (3 ± 1)</td>
<td>121 ± 10*</td>
</tr>
<tr>
<td>Day 11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>156 ± 4 (89 ± 2)</td>
<td>20 ± 4 (11 ± 2)</td>
<td>—</td>
<td>176 ± 6</td>
</tr>
<tr>
<td>GM-CSF sense</td>
<td>162 ± 12 (88 ± 1)</td>
<td>22 ± 2 (12 ± 1)</td>
<td>—</td>
<td>184 ± 11</td>
</tr>
<tr>
<td>GM-CSF antisense</td>
<td>76 ± 11 (72 ± 6)†</td>
<td>30 ± 1 (28 ± 6)†</td>
<td>—</td>
<td>106 ± 8†</td>
</tr>
<tr>
<td>GM-CSF-R sense</td>
<td>153 ± 9 (89 ± 2)</td>
<td>19 ± 2 (11 ± 2)</td>
<td>—</td>
<td>172 ± 5</td>
</tr>
<tr>
<td>GM-CSF-R antisense</td>
<td>91 ± 9 (76 ± 3)†</td>
<td>29 ± 1 (34 ± 3)†</td>
<td>—</td>
<td>120 ± 10†</td>
</tr>
</tbody>
</table>

Values are the means ±SD of four replicate cultures with percentage of total in parentheses.

* At day 7, significance at P ≤ .02 when compared with sense control.
† At day 11, significance at P ≤ .01 when compared with sense control.
‡ At day 11, significance at P ≤ .005 when compared with sense control.
the chromosomal gene for granulocyte-macrophage colony stimu-
corresponding group with sense oligomers. Both antisense
Both sense
GM-CSF antisense
GM-CSF sense
Epo antisense
Epo sense
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by activated TH type 2 cells. Its role as an autocrine factor. J Im-
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Specific repression of granulocyte-macrophage and granulocyte col-
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endothelial cells with antisense oligodeoxynucleotides. Blood
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### Table 7. Effect of Both Epo and GM-CSF Antisense Oligomers on 7-Day Colony Formation

<table>
<thead>
<tr>
<th></th>
<th>Mixed Non-E</th>
<th>Mixed E:None-E</th>
<th>BFU-E</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>84 ± 1 (73 ± 7)</td>
<td>26 ± 5 (23 ± 6)</td>
<td>5 ± 2 (4 ± 2)</td>
<td>115 ± 5</td>
</tr>
<tr>
<td>Epo sense</td>
<td>79 ± 0 (77 ± 4)</td>
<td>21 ± 3 (21 ± 3)</td>
<td>2 ± 1 (2 ± 1)</td>
<td>102 ± 8</td>
</tr>
<tr>
<td>Epo antisense</td>
<td>90 ± 0 (81 ± 4)*</td>
<td>15 ± 6 (12 ± 5)*</td>
<td>7 ± 2 (6 ± 1)</td>
<td>113 ± 11</td>
</tr>
<tr>
<td>GM-CSF sense</td>
<td>88 ± 0 (75 ± 2)</td>
<td>26 ± 2 (22 ± 2)</td>
<td>3 ± 1 (3 ± 1)</td>
<td>115 ± 5</td>
</tr>
<tr>
<td>GM-CSF antisense</td>
<td>45 ± 3 (64 ± 3)*</td>
<td>35 ± 4 (42 ± 2)*</td>
<td>4 ± 1 (5 ± 1)</td>
<td>84 ± 6*</td>
</tr>
<tr>
<td>Both sense</td>
<td>95 ± 17 (74 ± 5)</td>
<td>28 ± 4 (22 ± 4)</td>
<td>4 ± 1 (4 ± 1)</td>
<td>127 ± 16</td>
</tr>
<tr>
<td>Both antisense</td>
<td>58 ± 11 (74 ± 4)</td>
<td>15 ± 3 (18 ± 3)</td>
<td>6 ± 1 (8 ± 2)</td>
<td>79 ± 11*</td>
</tr>
</tbody>
</table>

* For both mixed nonerythroid colonies and mixed E:non-E colonies, each group with antisense oligomers differed significantly (P < .04) from the corresponding group with sense oligomers.
Further study of internal autocrine regulation of multipotent hematopoietic cells

N Pech, O Hermine and E Goldwasser