Antisense Oligonucleotides from the Stage-specific Myeloid Zinc Finger Gene MZF-1 Inhibit Granulopoiesis In Vitro

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Summary

Zinc finger proteins are transcriptional regulators of other genes, often controlling developmental cascades of gene expression. A recently cloned zinc finger gene, MZF-1, was found to be preferentially expressed in myeloid cells. Using complementary radiolabeled MZF-1 RNA hybridized to human bone marrow smears in situ, it was discovered that the expression of MZF-1 is essentially limited to the myelocyte and metamyelocyte stages of granulopoiesis. Antisense but not sense oligonucleotides from MZF-1 significantly inhibited granulocyte colony-stimulating factor–driven granulocyte colony formation in vitro.

With the fundamental discovery that bone marrow could be grown as colonies in vitro, the environmental signals that control blood cell development have become fairly well established. Most of these signals, called CSFs, have been cloned, as have many of their receptors (1–4). Indeed, two of these CSFs, granulocyte CSF (G-CSF)[1] and granulocyte/macrophage CSF (GM-CSF), have now been approved for use in clinical medicine.

However, the genetic program the CSFs induce is less well understood. From what is known about development in general, several assumptions can be made about the genetic control of hematopoiesis. Much of the developmental gene regulation occurs at the transcriptional level (5). Thus, it is likely that the genes that control hematopoiesis are for the most part transcription factors, and transcription factors fall into families based on shared amino acid sequences (6). Novel blood cell–specific transcription factors are candidates for the role of hematopoietic developmental regulators.

One such candidate is the zinc finger gene MZF-1, which is responsive to retinoic acid and preferentially expressed in myeloid cells (7). Zinc finger proteins are transcriptional regulators of other genes, and control morphogenesis (8).

Zinc finger genes may be important in blood cell development. GATA-1 is the prototype of a zinc finger family that controls globin expression in the developing red blood cell (9). Another hematopoietic zinc finger gene, EVI-1, appears to be responsible for the escape from CSF dependence by a murine myeloid leukemia (10).

In this study we used radiolabeled complementary RNA (cRNA) hybridized in situ to normal human marrow smears to assess the lineage and stage expression of MZF-1. Upon finding that MZF-1 was expressed mainly in the myelocyte and metamyelocyte stage in the granulocytic lineage, we performed G-CSF-driven granulocyte colony formation assays in the presence of sense and antisense synthetic deoxyoligonucleotides. Antisense oligonucleotides have been shown to be taken up by cells and to stimulate the degradation of complementary sequences by intracellular RNase H (11, 12). Such a technique has been used to demonstrate that c-*abl*, c-*myb*, and CD45 are important in hematopoietic colony formation in vitro (13–15). In this investigation, it was found that MZF-1 antisense but not sense oligonucleotides differentially inhibited granulocyte but not erythrocyte colony formation.

Materials and Methods

CRNA In Situ Hybridization. A partial cDNA clone of MZF-1, designated ZNF 1.6 (1.6 kb in length; 7), was subcloned into the EcoRI site of pGEM-2 (Promega Biotec, Madison, WI). To obtain radiolabeled cRNA, this vector was linearized with Sph 1, giving a probe length of 357 ribonucleotides from the T7 promoter. For control sense radiolabeled RNA, the vector was linearized with BamH1, giving a total probe length of 197 ribonucleotides. Radiolabeled RNA was generated as specified by Promega Biotec with α-S^{35}-UTP used as the label. Specific activity of the probes generated always exceeded 10^9 cpm/μg. There was usually >70% incorporation of the label into the probe.

To generate the marrow templates for hybridization, human bone marrow was aspirated into heparinized syringes from the posterior

[1] *Abbreviations used in this paper:* G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor.

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iliac crests of paid healthy adult volunteers after obtaining written consent according to a protocol approved by the Institutional Review Board at the University of Washington Medical Center. Low-density mononuclear cells were isolated by centrifugation in Ficoll-Hypaque (Organon Teknika, Durham, NC). The interface cells were collected, washed twice in HBSS (Gibco Laboratories, Grand Island, NY), and depleted of adherent cells by overnight double-plastic adherence in IMDM (Gibco Laboratories) supplemented with 10% FCS. The resulting nonadherent marrow cells were harvested by centrifugation, counted for viability using 0.5% trypan blue, and used directly for seeding of colony-forming assays or cytospin slide preparation for cRNA hybridization.

Low-density nonadherent marrow cells were cytospun directly onto poly-L-lysine-subbed glass slides and fixed immediately in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 4 min at 4°C, and stored in molecular grade 70% ethanol at 4°C. Care was taken to ensure the freedom from RNase contamination of all reagents.

In situ hybridization took place essentially as described (16). Slides were post-fixed for 10 min in 4% paraformaldehyde, rinsed 5 min in 0.5× SSC (1× = 0.15 M NaCl/0.15 M Na Citrate, pH 7.0), and immersed in proteinase K (1 μg/ml) for 10 min at room temperature. Unless otherwise indicated, all SSC solutions also contained 0.01 M 2-ME and 0.001 M EDTA to help prevent nonspecific binding of the ^{35}S-labeled probe. Slides were then washed in 0.5× SSC for 10 min, the glass surrounding the cytospun cells was dried with tissue paper, and then 50 μl of hybridization buffer (0.3 M NaCl, 0.02 M Tris, pH 8.0, 0.005 M EDTA, 0.01 M dithiothreitol, 10% dextran sulfate, 1% Ficoll, 1% polyvinyl pyrrolidone, and 1% albumin) was applied to the cytospin. The slides were preincubated without probe for 4 h at 42°C in airtight boxes containing filter paper saturated with 4× SSC and 50% formamide. 3 × 10^5 cpm of the probe in an additional 10 μl of hybridization solution with 10 μg of yeast tRNA was mixed into the hybridization buffer on the cytopsins. The boxes were resealed and incubated at 50°C for 24 h.

At the end of hybridization, slides were washed twice for 10 min in 2× SSC at room temperature, followed by digestion of free probe by immersion in 0.5 M NaCl containing 20 μg/ml of RNase A for 30 min at room temperature. Slides were then washed twice for 30 min at room temperature in 2× SSC, followed by a high stringency wash at 42°C in 0.1× SSC for 30 min. Slides were then washed twice in 2× SSC without 2-ME and EDTA for 10 min at room temperature, and dehydrated by immersion in graded alcohols containing 0.3 M ammonium acetate. After air drying slides overnight, they were dipped in the dark into Kodak NTB-2 autoradiographic emulsion at a 1:1 dilution in distilled water. The slides were then dried vertically for 30 min at room temperature, and exposed in desiccant-containing light-sealed boxes at 4°C for 28 d. They were then developed for 4 min in 1:1 Kodak D19 developer in distilled water, washed briefly in distilled water, followed by 5 min in Kodak Ektaflo fixer, all at room temperature. After development, slides were counterstained with hematoxylin unless otherwise indicated. Slides were photographed with a light microscope (E. Leitz, Inc., Rockleigh, NJ).

Oligodeoxynucleotide Effect on Marrow Progenitor Colony Formation. Preparation and addition of the synthetic oligodeoxynucleotides was carried out as described (14). The nonadherent low-density marrow cells were incubated in serum-free IMDM containing 1% BSA with the appropriate concentration of oligomer for 4 h shaking at 37°C. Cells were then plated at 10^5 in 1 ml of IMDM containing 0.8% methylcellulose, 20% FCS, 1% BSA, the appropriate oligomer concentration, and 1% penicillin/streptomycin/amphotericin (Gibco Laboratories) in 35-mm culture dishes as previously described (17). Since serum contains nucleases, appropriate concentrations of fresh deoxynucleotides were added 3 d later, and 5 d later (14). Additive oligomer and control volumes were normalized to 10 μl sterile distilled water per plate. Granulocyte colonies (CFU-G) were stimulated with a final concentration of 1 nM recombinant human G-CSF (Amgen, Thousand Oaks, CA). Erythroid bursts (BFU-E) were driven with 1 U/ml of recombinant human erythropoietin (18). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Granulocyte colonies were counted at day 10 and erythroid bursts at day 14 in a blinded fashion by duplicate personnel using an inverted microscope. Experiments were repeated at least twice with triplicate or quadruplicate plates per assay condition. Data are expressed as the mean number of colonies per 10^5 marrow cells plus or minus the SE.

Oligomers were designed in pairs, designated S for sense or AS for antisense orientation. We tested three pairs of oligomers, none of which had significant homology to any other nucleic acid sequence in GenBank release 59.0. Oligomer pair no. 1 came from nucleotide 1726–1749, pair no. 2 From 2384–2402, and pair no. 3 from 2038–2053 of the MZF-1 cDNA sequence (GenBank/EMBL accession number M58297, 7).

Results

We performed in situ cRNA hybridization of normal human marrow to establish the lineage and stage of expression of MZF-1. Fig. 1 shows four typical micrographs of the hybridized, counterstained marrow smears. Silver grains overlying cells indicate specific hybridization of the probe to MZF-1 mRNA in that cell. Although background was rarely more than one grain per cell, cells were not counted as positive unless they had five or more grains overlying them.

Analysis of the marrow hybridization patterns revealed that only cells of the granulocyte lineage expressed MZF-1. Neither lymphocytes, megakaryocytes, or erythrocytes expressed MZF-1. In addition, no myeloblasts or mature granulocytes could be found to be positive despite extensive microscopic searches. MZF-1 appeared to be expressed in cells transversing the myelocytic or metamyelocyte stage. Fig. 1 *A* shows two late myelocytes overlaid with silver grains. Fig. 1, *B* and *C* show a broader field of hematopoietic cells, with the positive myelocytes and metamyelocytes surrounded by negative erythrocytes, granulocytes, and lymphocytes.

Counting 100 positive cells in a random marrow smear revealed that of those cells expressing MZF-1, 2% were promyelocytes, 46% myelocytes, and 52% metamyelocytes.

HL60 cells induced to differentiate towards granulocytes with 48 h of 10^{-6} M retinoic acid served as the positive control. Almost every cell expressed MZF-1 as indicated by the overlying silver grains (Fig. 1 *D*). Marrow smears hybridized with sense as opposed to complementary (antisense) radiolabeled RNA served as negative controls (Fig. 1 *E*). No cells specifically hybridized to this probe.

To ascertain whether MZF-1 expression was important in granulocyte development, we performed granulocyte colony formation assays in vitro in the presence of sense or antisense synthetic deoxyoligonucleotides. The effect of MZF-1 antisense and sense oligonucleotides on granulocyte colony formation

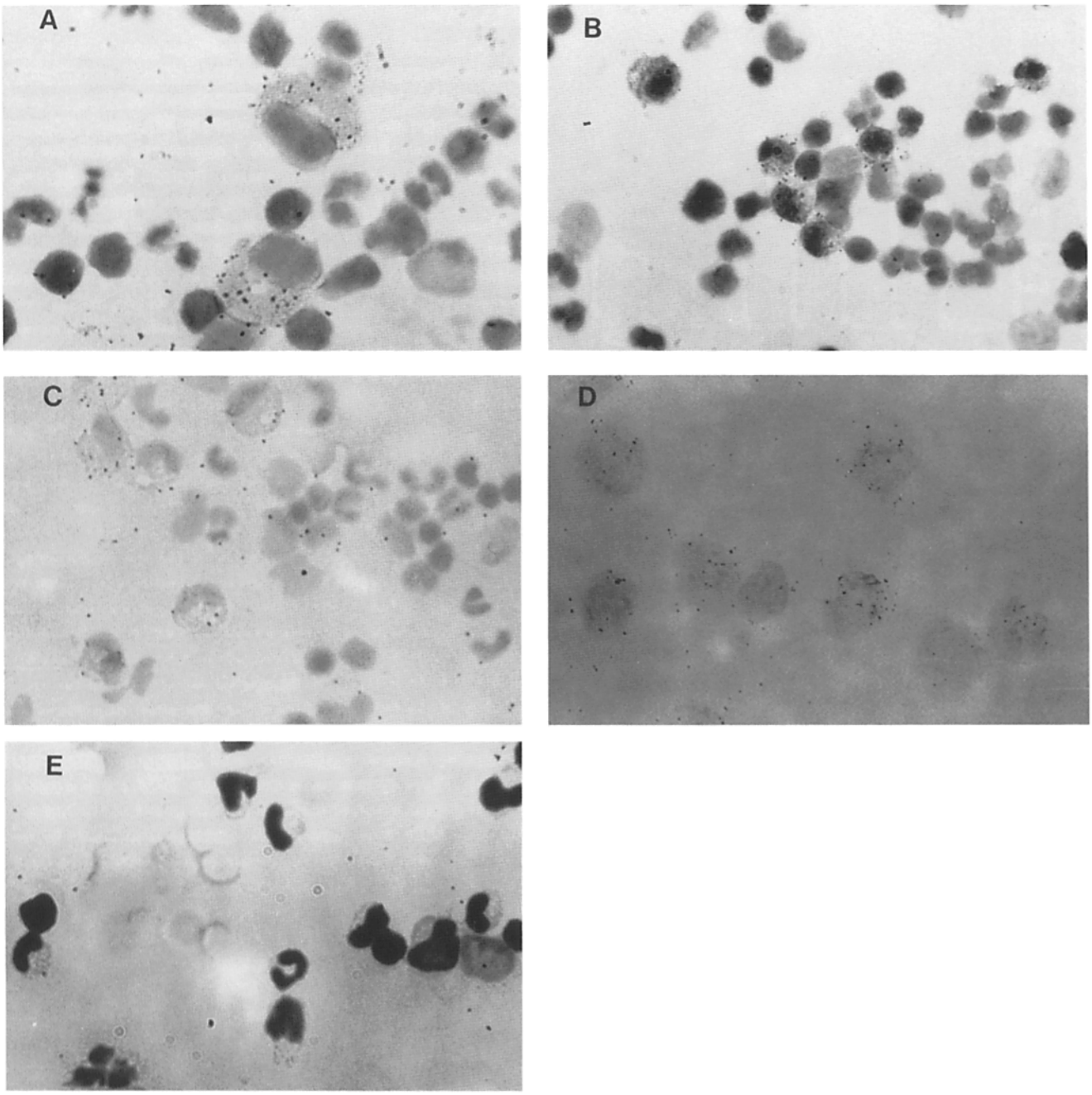

Figure 1. Demonstration of the marrow cell type specificity of the myeloid zinc finger gene, MZF-1, using in situ cRNA hybridization. (*A*) High-power photomicrograph of marrow cells radiolabeled with MZF-1 cRNA. Cells with more than five silver grains overlying them were counted as positive. (*B*) Low-power photomicrograph of a different field of marrow cells hybridized to MZF-1 cRNA. (*C*) Low power photomicrograph of a separate field stained with Romanowsky stain. (*D*) Retinoic acid–treated HL60 cells hybridized in situ with MZF-1 cRNA as a positive control. This slide was counterstained with Romanowsky stain. (*E*) Marrow cytospins hybridized to radiolabeled MZF-1 sense RNA as a negative control. Although this slide was overstained, the overlying silver grains are clearly discernable as nonspecific.

is shown in Fig. 2. The three sense oligomers had little effect on granulocyte colony formation, while the three antisense oligomers appeared to have significant inhibitory activity. In one of the experiments that makes up Fig. 2, the colonies were counted at 7 d in addition to 10 d. Although the colonies were less than our standard of 50 cells, the data obtained were equivalent to 10 d. There was no increase in eosinophil colonies in any of these cultures over controls. The two antisense oligomers having the most inhibitory activity and their sense counterparts, oligomer pair nos. 1 and 2, were tested for a dose-response effect on granulopoiesis (Fig. 3). At the highest oligomer concentration, 24 μM, antisense oligomer 1 lowered CFU-G from the sense control value of 106 ± 5.0% to 7.6 ± 0.5%, while antisense oligomer 2 lowered

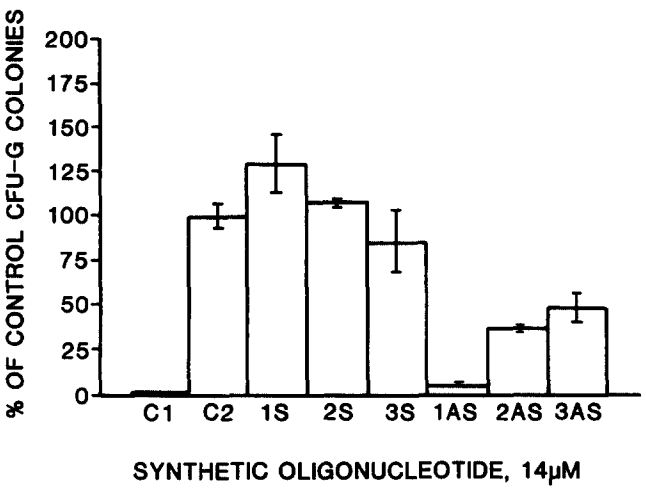

Figure 2. Effect of synthetic deoxynucleotides on granulocyte colony formation in vitro. C1 and C2 are the percent growth of CFU-G without and with G-CSF as stimulant, respectively. Three oligomer pairs were tested. S represents the sense, and AS the antisense orientation of the single stranded oligomers. The C2 group averaged 136 colonies per plate.

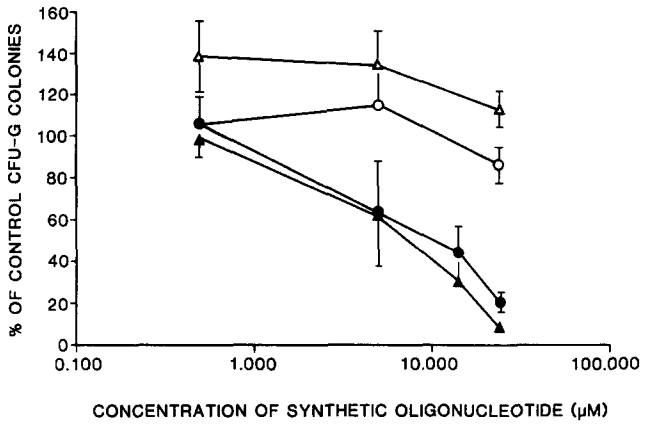

Figure 3. A dose-response curve of the effect of synthetic single-stranded deoxyoligonucleotides on granulocyte colony formation in vitro. The open symbols represent oligomers in the sense orientation, while the closed symbols the antisense orientation. The triangles represent oligomer pair no. 1 while the circles represent oligomer pair no. 2. In these experiments, the G-CSF control plates averaged 81 colonies.

CFU-G from 80 ± 5.5% to 18 ± 2.2%. Interestingly, the colonies that did appear after treatment with the antisense nucleotides generally were smaller. Upon cytospin preparation and Wright's staining these colonies, the cells appeared dysplastic as compared to the sense controls.

When oligomer pairs 1 and 2 were used to treat erythropoietin-driven BFU-E, there was a minimal difference between the sense and antisense form in inhibitory activity. However, both sense and antisense oligomers were somewhat toxic to the BFU-Es. At 14 μM, oligomer 1AS had 64 ± 12% of control BFU-E formation, while 1S had 78 ± 3%. Oligomer 2AS had 61 ± 9.1%, while 2S had 75 ± 13%. For these experiments, control assays averaged 36 colonies per culture dish.

Discussion

The recently cloned human zinc finger gene, MZF-1, was found to be preferentially expressed in HL60 cells and markedly induced when those cells were stimulated to differentiate towards granulocytes with retinoic acid (7). This study addressed two questions. First, was MZF-1 expression restricted to a specific lineage or stage in normal human hematopoiesis? Second, was MZF-1 expression important in hematopoiesis?

Using radiolabeled cRNA hybridization of normal human marrow, it was found that MZF-1 expression was limited mainly to myelocytes and metamyelocytes in the granulocytic lineage. No expression could be found in the erythroid, lymphoid, or megakaryocytic lineages. Northern analysis had found that besides HL60 cells, MZF-1 was expressed to a lesser extent in HEL and K562 cells, human erythroleukemia lines, and in KG-1 cells, a myeloid leukemia line. In the present study, neither erythroblasts nor myeloblasts expressed MZF-1. It may be that expression was too low to be detected in our assay; although the uniformly radiolabeled cRNA probes had very high specific activity. Another possibility is that normal blast progenitors do not express MZF-1. The above cell lines may also represent earlier precursors than the morphologically identifiable erythroblasts and myeloblasts in the marrow.

In the second part of this investigation, it was found that antisense synthetic deoxynucleotides to MZF-1 inhibited in vitro human CFU-G formation while having minimal effect on BFU-E formation as compared to sense oligomers. A number of studies have shown that antisense oligomers can be taken up by the cell, and degrade specific mRNA, preventing protein expression and changing phenotypes (11–15). These experiments imply that MZF-1 has a role in normal granulopoiesis. More definitive proof of the requirement for MZF-1 in granulocytic development could be uncovered in future experiments overexpressing MZF-1 in stably transfected granulocytic precursors. In addition, this study does not delineate exactly which granulocyte precursor is inhibited by antisense MZF-1. Further investigations are under way using flow cytometrically sorted marrow progenitors examining the role MZF-1 may have in their development.

There are three other zinc finger genes that are important in hematopoiesis, GATA-1 is the prototype of a family of zinc finger genes that stimulate globin transcription in red blood cell development (9). Deleting the GATA-1 gene in the germline of mice by homologous recombination inhibited erythropoiesis (19). The second hematopoietic zinc finger gene is EVI-1. Expression of this gene allows murine myeloid cells to escape dependance on IL-3 (10). Finally, the retinoic acid receptor α is disrupted and fused to a novel transcribed locus in most cases of acute promyelocytic leukemia (20). Treating these patients with all-*trans* retinoic acid induces complete remissions of the leukemia (21). This is perhaps the most convincing evidence yet that the genetic cascade of hematopoietic differentiation might be controlled in part by transcription factors such as zinc finger proteins. From the data presented in this study, MZF-1 may be another such gene, playing a role in stage progression during granulocyte development.

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