Release of Early Human Hematopoietic Progenitors from Quiescence by Antisense Transforming Growth Factor β1 or Rb Oligonucleotides

By Jacques Hatzfeld,* Ma-Lin Li,* Eugene L. Brown,‡ Hemchand Sookdeo,‡ Jean-Pierre Levesque,* Timothy O'Toole,‡ Carol Gurney,‡ Steven C. Clark,‡ and Antoinette Hatzfeld*

*From the *Laboratoire C.N.R.S. de Biologie Cellulaire et Moléculaire des Facteurs de Croissance, ICIG, Hôpital Paul-Brousse, 94802 Villejuif Cedex, France; and ‡Genetics Institute, Inc., Cambridge, Massachusetts 02140*

Summary

We have used antisense oligonucleotides to study the roles of transforming growth factor β (TGF-β) and the two antioncogenes, retinoblastoma susceptibility (Rb) and p53, in the negative regulation of proliferation of early hematopoietic cells in culture. The antisense TGF-β sequence significantly enhanced the frequency of colony formation by multi-lineage, early erythroid, and granulomonocytic progenitors, but did not affect colony formation by late progenitors. Single cell culture and limiting dilution analysis indicated that autocrine TGF-β is produced by a subpopulation of early progenitors. Antisense Rb but not antisense p53 yielded similar results in releasing multipotential progenitors (colony-forming unit–granulocyte/erythroid/macrophage/megakaryocyte) from quiescence. Rb antisense could partially reverse the inhibitory effect of exogenous TGF-β. Anti-TGF-β blocking antibodies, antisense TGF-β, or Rb oligonucleotides all had similar effects. No additive effects were observed when these reagents were combined, suggesting a common pathway of action. Our results are consistent with the model that autocrine production of TGF-β negatively regulates the cycling status of early hematopoietic progenitors through interaction with the Rb gene product.

Among the normal hematopoietic progenitor populations, the earliest pluripotent cells are largely quiescent, while a larger proportion of later, lineage-restricted cells are in active phases of the cell cycle (1). Relatively little is known about the mechanisms that maintain early cells in a quiescent state (2–6), although recent reports have demonstrated that TGF-β is a potent and specific inhibitor of colony formation by early hematopoietic progenitors (7–9). In skin keratinocytes and in lung epithelial cells, the growth inhibition of TGF-β has been linked to its ability to prevent the inactivation of the product of the growth suppressive retinoblastoma susceptibility gene, RB (10, 11). Other reports suggest that the TGF-β and Rb genes might be involved in multiple pathways of cellular growth control (12, 13). However, it is difficult to purify a sufficient number of early human bone marrow progenitors to perform molecular analysis of these pathways. Therefore, to study the roles of these two gene products in controlling the cycling status of early human hematopoietic progenitors, we have used antisense oligonucleotides in single cell and clonal cultures of enriched progenitors. Our results indicate that the autocrine production of TGF-β negatively regulates the cycling status of early hematopoietic progenitors and, with at least some cell populations, this regulation is mediated by interaction with the Rb gene product.

Materials and Methods

Growth Factors and Antibodies. Granulocyte (G)-CSF, IL-3, and IL-6 were from Genetics Institute (Cambridge, MA), and Epo from Integrated Genetics (Framingham, MA). TGF-β blocking antibody for the type 1 isoform was raised in turkeys and was a generous gift of Drs. A. B. Roberts, and M. B. Sporn (14). 1 μl could neutralize 4 ng of TGF-β1. TGF-β1 was a generous gift of Dr. D. A. Lawrence (Institut Curie, Orsay, France).

Bone Marrow. Specimens of human bone marrow were obtained either from normal bones at orthopedic surgery or from normal bone marrow transplant donors with their informed consent. All samples were collected on heparin.

Cell Preparation and Cell Culture. Human bone marrow cell progenitors were prepared as follows: $CD34^+$ cells were enriched by one passage on a soybean agglutinin CELLector flask to remove mature cells and one passage of the nonagglutinated cells on a ICH3 CD34 antibody-covered CELLector flask (Applied Immune Sciences Inc., Menlo Park, CA), following the instructions of the manufacturer. $CD34^+$ cells were tested according to a modification of the mixed colony assay of Fauser and Messner (15): cells were plated in methylcellulose with 30% FCS, 1.7 U/ml IL-3, 10 U/ml IL-6, 4 U/ml G-CSF, and 1.5 U/ml Epo either in 35-mm non–culture-treated Petri dishes with 10^2 to 3.10^3 cells per ml, or in single cell cultures in the wells of 96-well plates. Cultures were incubated for 14 d at 37°C in humidified atmosphere containing 5% CO_2 in air. For 10^3 cells plated in 1 ml of methylcellulose culture medium,

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we obtained: 75 ± 6 monocytic colonies, 86 ± 7 granulocytic colonies, 17 ± 3 granulomonocytic colonies, 2 ± 2 erythroid clusters (CFU-E), 25 ± 3 erythroid burst (BFU-E), 37 ± 5 large erythroid burst (early BFU-E, containing sometimes rare megakaryocytes), and 4 ± 1 mixed colonies (CFU-GEMM).

For single cell experiments, cells were first plated at 10^3 cells per 35-mm Petri dish and then picked separately with a Pasteur pipette under an inverted microscope and plated in 50 μl of culture medium per well of a 96-well plate.

Colony Classification. Colonies were classified according to established criteria (16) by direct observation of the dishes with an inverted microscope (E. Leitz Inc., Wetzlar, FRG).

Oligonucleotides. For our studies, we designed 21mers corresponding to the sense or antisense sequences flanking the translation initiation regions of the mRNAs for TGF-β1, Rb1, and p53. These oligonucleotides were synthesized using phosphorothioate linkages because of their demonstrated resistance to nucleases (17).

The sequence of the phosphorothioate oligonucleotides are as follows with the ATG initiation codon or its complement CAT underlined in the sense and antisense sequences, respectively: TGF-β antisense, 5'-CCCGGAGGGCGGCATGGGGGA-3'; TGF-β sense, 5'-TCCCCCATGCCGCCCTCCGGG-3'; TGF-β missense, 5'-GGCGAGCGAGTGAGCGCGCGG-3'; Rb antisense, 5'-GTGAACGACATCTCATCTAGG-3'; Rb sense, 5'-GATGAGATGTCGTTCACTTTA-3'; Rb missense, 5'-AGCTAGCTAGCTAGCTAGCTA-3'; p53 antisense, 5'-CTGCGGCTCCTCCATGGCAGT-3'; p53 sense, 5'-ACTGCCATGGAGGAGCGCAG-3'. Phosphorothioate oligonucleotides were prepared on a DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) with nucleoside 3'-hydrogen phosphonates. At the end of the synthesis cycle, the full length H-phosphonate oligonucleotides were converted to the thioate analogues with a sulfurization protocol supplied by Applied Biosystems, Inc. The crude products were purified by HPLC on a C-18 reverse phase column with a linear gradient of acetonitrile in 50 mM triethylammonium acetate. Computer searches did not reveal any significant sequence similarity between the different 21mers and any of the sequences in GenBank, including TGF-β2 and TGF-β3.

Preliminary experiments with radiolabeled oligonucleotides demonstrated that these short sequences enter the various types of cells equally well. We did not observe any difference between the effects of the sense and missense oligonucleotides (not shown).

Statistical Analysis. The mean of the values ± SD for different experiments are shown in the figures and in Table 1. Significant differences between treatment groups were determined by using Student's *t* test applied for paired samples.

Results and Discussion

As illustrated in Fig. 1, addition of the antisense but not the sense TGF-β oligonucleotide resulted in a dose-dependent increase in the formation of hematopoietic colonies from cultures of enriched progenitors. In subsequent experiments, the various oligonucleotides were used at concentrations between 5 and 8 μM to avoid the toxic effect observed at concentrations >10 μM.

That the antisense TGF-β oligonucleotide enhanced colony formation from different types of progenitor cells is shown in Fig. 2. CD34$^+$ progenitors were plated under optimal growth conditions as single cell cultures in the wells of a 96-well plate or at low cell concentration in 35-mm Petri dishes. In the presence of 5 μM antisense oligonucleotide,

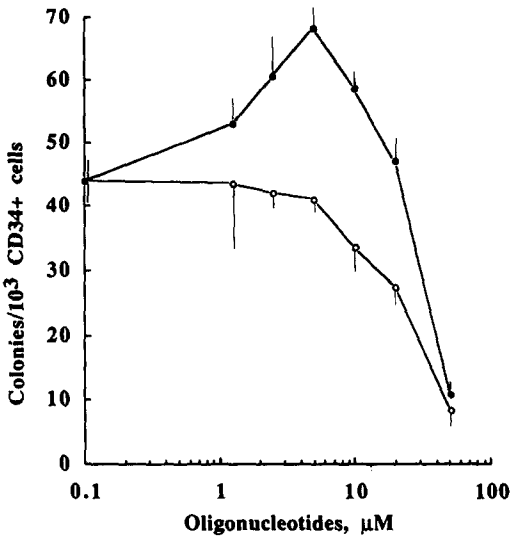

Figure 1. Antisense TGF-β enhancement of colony formation by CD34$^+$ human progenitors. The indicated concentrations of antisense (●) or sense (○) TGF-β oligonucleotides were added to cultures of CD34$^+$-enriched human progenitors. Total colonies derived from CFU-GEMM, CFU-GM, BFU-E, CFU-G, and CFU-M were enumerated after 12–14 d in culture.

twice as many mixed colonies derived from the multipotential progenitors CFU-GEMM were obtained, as compared to control cultures with TGF-β sense ($p < 0.001$, df = 11). The resulting colonies were also larger on average than those obtained in control cultures (1.6–3.2 × 10^4 vs. 0.8–1.6 × 10^4 cells/colony). The antisense-containing cultures also yielded 1.5–2.0-fold more erythroid (E) colonies derived from

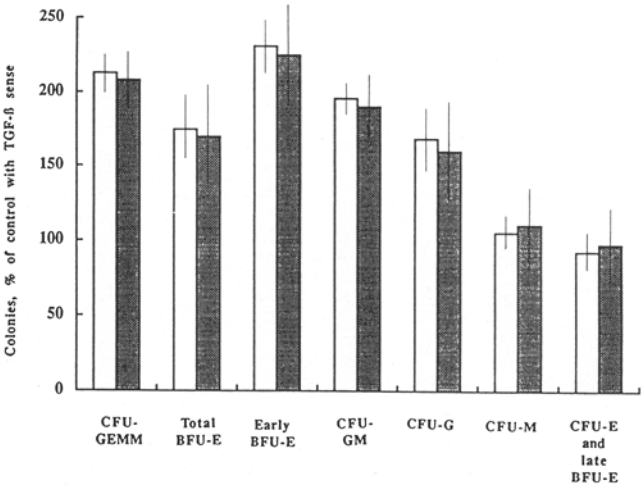

Figure 2. TGF-β antisense enhancement of colony formation by different types of progenitors plated at low cell density or in single cell culture. 5 μM TGF-β sense or antisense oligonucleotides was included in cultures of CD34$^+$ bone marrow cells plated at 10^3 cells/ml (□) or in single cell culture (▨) in the wells of 96-well plates. Colony counts are reported as the percentage of the respective colonies obtained with antisense as compared to colonies obtained with sense oligonucleotides. Cultures with sense oligonucleotides were similar to control cultures without oligonucleotides.

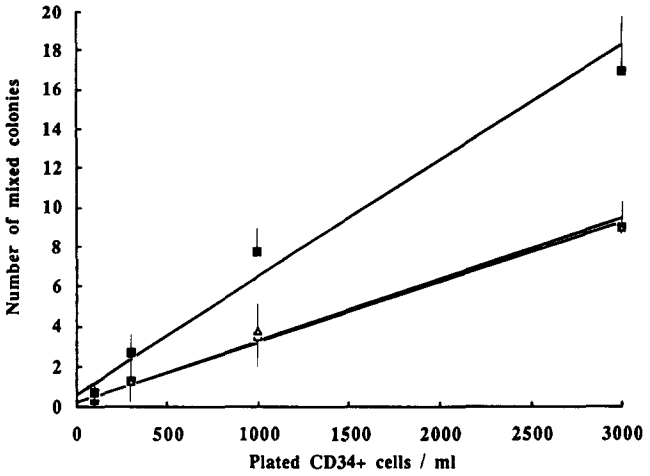

Figure 3. Effect of cell density on the frequency of mixed colony formation by $CD34^+$ cells. Progenitor cells were enriched and plated at different cell densities as described in Fig. 1 in the presence of 5 μM antisense (■) or sense (□) TGF-β oligonucleotides or in the absence of oligonucleotides (Δ). The data represent the mean of the mixed colony counts from 20, 10, 6, and 2 dishes of cultures containing 100, 300, 1,000, and 3,000 $CD34^+$ cells/ml, respectively. Least square regression analysis yielded Y intercepts of 0.588 ± 0.499 (■), 0.216 ± 0.351 (□), and 0.227 ± 0.403 (Δ) (values ± 95% confidence interval, df = 36, t = 2.03).

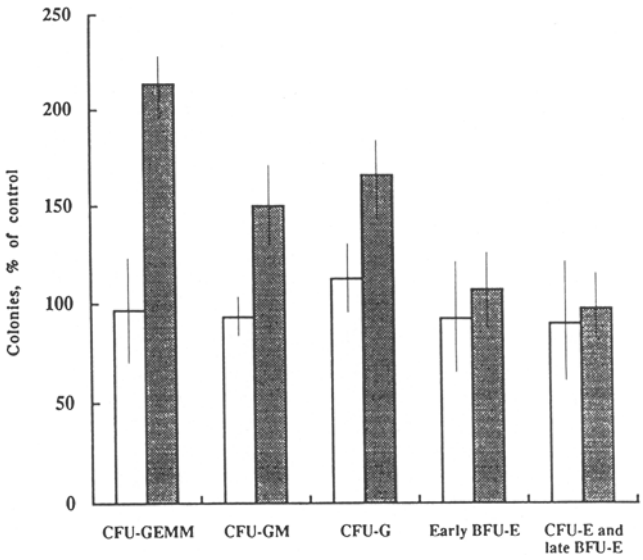

Figure 4. Rb antisense oligonucleotide enhancement of colony formation by different types of progenitor cells. 8 μM antisense (▨) or sense (□) Rb oligonucleotides were added to cultures of $CD34^+$ progenitors cells as described in Fig. 1. Colony counts, enumerated after 14 d in culture, are reported as the percentage of the respective colony types obtained in the absence of oligonucleotides.

early erythroid progenitors (BFU-E) and a similar increase of granulocyte-monocyte (GM) and granulocyte (G) colonies ($p < 0.005$, $9 \leqslant df \leqslant 11$). The number of very large erythroid colonies derived from the earliest BFU-E was even increased 2–4.5-fold. In contrast, TGF-β antisense had no effect on late erythroid progenitors (CFU-E and late BFU-E) nor on macrophage colony formation, whether or not macrophage colony-stimulating factor (M-CSF) was included in the culture (data not shown). Finally, although the antisense oligonucleotide significantly increased the frequency of large granulocyte colonies in cultures supplemented with both IL-3 and G-CSF, it had no effect on small ones typically obtained in cultures maintained with G-CSF alone (data not shown).

Fig. 2 shows no significant difference between single cell and low cell density cultures, except that the colonies were larger in 35-mm plates, probably due to poor gas exchange in the 96-well plates. These results demonstrate that the various types of progenitors (CFU) are single cells and that the resulting colonies are clonal and do not depend on accessory cells for their response to growth factors or oligonucleotides. This is further demonstrated in Fig. 3, which shows that the frequency of mixed colonies was linearly related to the input $CD34^+$ cell number when tested at concentrations as low as 100 cells in a 1-ml culture. When extrapolated to 0 input cells, the best line fit of the data of Fig. 3 originates very close to the X/Y intersection (0 input, 0 colonies). These results demonstrate that early progenitors themselves and not accessory cells produce the negative regulatory molecule, TGF-β.

The success of antisense TGF-β in releasing hematopoietic progenitors from a quiescent, growth factor–unresponsive state prompted us to try a similar approach for blocking the expression of two intracellular regulators of cell proliferation, p53 and Rb. Although the antisense p53 oligonucleotide did not enhance colony formation by any type of progenitor cell tested (data not shown), the Rb antisense oligonucleotide resulted in a twofold increase in the frequency of mixed colony (CFU-GEMM) formation ($p < 0.001$, df = 7) and an increase in granulo/monocytic and granulocytic colonies ($p < 0.05$, df = 5) grown in the presence of IL-3 and G-CSF (Fig. 4), but had no effect on G-CSF supported CFU-G (data not shown). In contrast to the antisense TGF-β, the antisense Rb oligonucleotide had no effect on BFU-E. Rb sense or missense oligonucleotides had a negligible toxic effect up to 8 μM (data not shown).

To test for possible interactions of TGF-β with the Rb gene product in the control of cycling of early hematopoietic cells, we examined the effects of combinations of TGF-β, anti-TGF-β antibody, or antisense TGF-β oligonucleotide with antisense Rb oligonucleotide on colony formation by CFU-GEMM from $CD34^+$ enriched human bone marrow cells (Table 1). In this experiment, addition of anti-TGF-β antibody yielded the same enhancement of CFU-GEMM colony formation as achieved with addition of either antisense TGF-β or Rb oligonucleotides, demonstrating that the autocrine TGF-β acts external to the cell. Combination of antibody against TGF-β with antisense Rb oligonucleotide did not result in additional enhancement of colony formation, indicating that these agents act on the same cells. Finally, addition of exogenous TGF-β to the cultures completely blocked CFU-GEMM colony formation, and this inhibition was partially reversed by the addition of antisense Rb oligonucleo-

Table 1. *Effects of TGF-β, Anti-TGF-β* Antibodies, Antisense TGF-β, *or Rb Oligonucleotides on Multipotential Progenitors (CFU-GEMM)*

Added factors	Mixed colonies/3 × 10^3 cells
Control	9 ± 2
TGF-β sense	8 ± 1
Rb sense	8 ± 1
TGF-β antisense	20 ± 2
Rb antisense	17 ± 3
Turkey irrelevant antiserum	9 ± 1
Anti-TGF-β antibodies	19 ± 3
Anti-TGF-β antibodies + Rb sense	17 ± 2
Anti-TGF-β antibodies + Rb antisense	18 ± 3
TGF-β	1 ± 1
TGF-β + Rb sense	0 ± 0
TGF-β + Rb antisense	7 ± 1

$CD34^+$ cells were enriched and cultured as described in Materials and Methods. TGF-β was added at 1 ng/ml. A turkey anti-TGF-β blocking antiserum was added at 0.75 μl/ml. Rb and TGF-β oligonucleotides were added at 8 and 5 μM, respectively. This is one experiment out of four similar ones.

tide, indicating that with at least some early cells, functional RB protein is required to mediate TGF-β growth inhibition.

Our results implicate TGF-β as an important negative autocrine growth regulator of several different types of hematopoietic progenitors, including the early multipotent CFU-GEMM but also the slightly more differentiated early BFU-E, CFU-GM, and early CFU-G. With some of these cell populations, more than half of the detectable colony formation progenitor cells are maintained in a growth factor unresponsive state by autocrine production of TGF-β.

The similar enhancement of CFU-GEMM, CFU-GM, and CFU-G colony formation achieved with antisense Rb oligonucleotides suggested that the inhibition of cycling of these cell types by TGF-β might be mediated through the Rb gene product, an expectation confirmed with at least some CFU-GEMM. Within the CFU-GEMM population in which the antisense Rb oligonucleotide released the TGF-β block, our results are consistent with other studies that have demonstrated that treatment of cells with TGF-β results in the accumulation of under-phosphorylated RB protein within the cell immediately before growth arrest. Inactivation of the RB protein through phosphorylation is believed to be a key step in allowing most if not all types of mammalian cells to cross the G1/S boundary and begin active cycling (18–20). However, it seems unlikely that inhibition of growth of hematopoietic cells by TGF-β and Rb will involve a simple linear sequence of events, because recent reports from other systems have shown multiple overlapping pathways mediated by these two gene products (12). For example, the RB protein itself can either induce or repress TGF-β expression, depending on the cell type (13). Our own data, in which antisense TGF-β but not antisense Rb oligonucleotides enhanced growth of early BFU-E, point out the complexity of the system. In this case, either the antisense Rb, in contrast to the antisense TGF-β, failed to work in this cell population, or the TGF-β inhibition is mediated through some other mechanism. It is perhaps relevant that several other proteins that appear to be functionally related to RB have also been described, and it will be of interest to see if any of these are involved in the control of the cycling of erythroid progenitors (21–23). The use of antisense oligonucleotides, which permits the study of the function of growth regulatory genes in rare cells that cannot be prepared in pure form in large quantities, should facilitate this analysis. Antisense oligonucleotides in the future may also prove useful in the amplification of normal hematopoietic stem cells through release of intracellular control mechanisms that prevent them from leaving G_0.

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Address correspondence to Jacques Hatzfeld, Laboratoire C.N.R.S. de Biologie Cellulaire et Moléculaire des Facteurs de Croissance, I.C.I.G., Hôpital Paul-Brousse, 94802 Villejuif Cedex, France.

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