

# Growth Factor-dependent Inhibition of Normal Hematopoiesis by N-ras Antisense Oligodeoxynucleotides

By Tomasz Skorski,\* Cezary Szczylik,\* Mariusz Z. Ratajczak,§  
Lucia Malaguarnera,\* Alan M. Gewirtz,§ and Bruno Calabretta†

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From the \*Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107; †Istituto Regina Elena, 00165 Roma, Italy; and the §Departments of Pathology and Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19103

## Summary

To determine whether N-ras expression is required at specific stages of the process of in vitro normal human hematopoiesis, adherent- and T lymphocyte-depleted mononuclear marrow cells (A-T-MNC) or highly purified progenitors (CD34<sup>+</sup> cells) were cultured in semisolid medium, under conditions that favor the growth of specific progenitor cell types, after exposure to N-ras sense and antisense oligodeoxynucleotides. N-ras antisense, but not sense, oligodeoxynucleotide treatment of A-T-MNC and CD34<sup>+</sup> cells resulted in a significantly decreased number of granulocyte/macrophage colony-forming units (CFU-GM) induced by interleukin 3 (IL-3) or granulocyte/macrophage colony-stimulating factor (GM-CSF) and of macrophage colonies (CFU-M) induced by M-CSF, but not of granulocytic colonies induced with G-CSF or IL-5. However, the same treatment significantly inhibited colony formation induced by each of the above factors in combination with IL-3. Megakaryocytic colony (CFU-Meg) formation from A-T-MNC or CD34<sup>+</sup> cells in the presence of IL-6 + IL-3 + erythropoietin (Epo) was also markedly decreased after antisense oligodeoxynucleotide treatment. Erythroid colonies derived from A-T-MNC in the presence of Epo (CFU-E) were not inhibited upon antisense treatment, whereas those arising from A-T-MNC or CD34<sup>+</sup> cells in the presence of IL-3 + Epo (BFU-E) were markedly affected. These results are consistent with the hypothesis that distinct signal transduction pathways, involving N-ras or not, are activated by different growth factors in different hematopoietic progenitor cells.

During the process of hematopoiesis, pluripotent hematopoietic stem cells generate lineage-specific progenitors that proliferate extensively and acquire a distinct phenotype before terminal differentiation into circulating myeloid and erythroid cells (1). This process is regulated by the coordinate activity of several hematopoietic growth factors, which, upon interaction with their membrane receptors, are likely to transmit the proliferation and/or differentiation activating signals from the cell surface membrane to the nucleus by activation of numerous signal-transduction pathways (2–5). Because some hematopoietic growth factors receptors have tyrosine kinase activity, or are associated with other tyrosine kinases (6–8), it seems likely that phosphorylation of tyrosine residues on target molecules is involved in this process. Oncogene products with constitutively enhanced tyrosine kinase activity activate the p21 ras protein (9), a member of a family of GTP-binding proteins that appear to be involved in transduction of growth signals inside the cell (10), via increased amount of the active GTP-bound protein.

The receptors for platelet-derived growth factor and epidermal growth factor transduce signals to initiate DNA synthesis through activation of p21 ras (11, 12). Stimulation of T lymphocyte proliferation by PHA, and their activation via CD3 complex or CD2 antigen, causes activation of p21 ras (13, 14). Increased levels of activated p21 ras, observed after treatment of several cell lines with IL-2, IL-3, and GM-CSF (15), suggest a role for p21 ras in the response of different cell types to growth factor stimulation. To determine whether N-ras is directly involved in the regulation of normal hematopoiesis, we studied the effects of inhibiting N-ras expression on normal hematopoietic proliferation. Our approach is based on the assumption that the generation of hematopoietic colonies in vitro closely reflects the process occurring in vivo under the influence of a variety of hematopoietic growth factors (5) and on the demonstration that antisense oligodeoxynucleotides can inhibit specific gene function in hematopoietic cells (16–18).

Our findings support the possibility that N-ras protoon-

cogene is involved in the regulation of human hematopoiesis through a differential growth factor-dependent activation in distinct progenitor cell subpopulations.

## Materials and Methods

**Cells.** Marrows were obtained by aspiration from the iliac crest of healthy volunteers after informed consent. Light-density mononuclear cells, separated on Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) density gradient, were enriched for hematopoietic progenitors after removing adherent cells and T lymphocytes (A-T-MNC)<sup>1</sup> as described (19). A-T-MNC were used as such or were further enriched for immature hematopoietic progenitor (CD34<sup>+</sup>) cells using HPCA-1 mAb (Becton Dickinson & Co., San Jose, CA) and Dynabead M-450 magnetic polystyrene beads coated with sheep anti-mouse IgG1 (Fc) (Dyna, Oslo, Norway) (20). The yield of CD34<sup>+</sup> cells from A-T-MNC ranged between 0.3 and 2.7% of the starting MNC population.

**Oligodeoxynucleotides.** These were synthesized on a DNA synthesizer (model 380B; Applied Biosystems, Inc., Foster City, CA) by means of  $\beta$ -cyanoethyl-phosphorymidite chemistry as described (18). The sequences of N-ras antisense and sense oligodeoxynucleotides used are 5'-CAG TTT GTA CTC AGT CAT -3' and 5'-ATG ACT GAG TAC AAA CTG -3'; respectively, which is complementary (or corresponding) to 18 nucleotides of exon 1 beginning with the initiation codon. N-ras mRNA was detected by amplification of a segment of N-ras exon 1 using the reverse transcription polymerase chain reaction (RT-PCR) technique with a 5' synthetic primer (5' ATC TCA TTT TAC TAG GGT GG 3') and a 3' synthetic primer (5' CTG GTG TGA AAT GAC TGA GT 3'). The amplification product was detected by hybridization to a synthetic oligomer included in the amplified segment (5' GTG GTG GTT GGA GCA GGT GGT GTT GGG AAA AGC GCA CTG 3') encompassing nucleotides 746–784 of the human N-ras cDNA (21).  $\beta_2$ -microglobulin ( $\beta_{2m}$ ) mRNA levels were also analyzed by RT-PCR. The 5' primer corresponds to nucleotides 280–301 of the  $\beta_{2m}$  mRNA sequence; the 3' primer corresponds to nucleotides 510–531; a 50-base probe used to detect the amplification product corresponds to nucleotides 351–400 (22).

**Oligomer Treatment of the Cells.** 10<sup>5</sup> A-T-MNC or 10<sup>4</sup> CD34<sup>+</sup> cells were seeded into 12 × 75-mm tubes (Falcon Labware, Oxnard, CA) in 0.4 ml of IMDM supplemented with 2% heat-inactivated human AB serum, recombinant human growth factors, Hepes buffer (Hazelton Biologics Inc.) and N-ras antisense or sense oligodeoxynucleotides. Recombinant human growth factors (Genetics Institute, Cambridge, MA) were IL-6 (125 U/ml), IL-3 (50 U/ml), GM-CSF (12.5 ng/ml), M-CSF (50 U/ml), IL-5 (1:1,000 crude conditioned medium from a CHO cell line constitutively expressing a human IL-5 cDNA), G-CSF (1:10,000 crude conditioned medium from a CHO cell line constitutively expressing a human G-CSF cDNA), and erythropoietin (Epo) (7.5 U/ml). Sense and antisense oligodeoxynucleotides were used at a concentration of 80  $\mu$ g/ml during the first 18 h of incubation. Then a second dose (40  $\mu$ g/ml) was added and cells were further incubated for 6 h and plated without washing. Control groups were left untreated.

**Colony Assays.** Duplicate cultures were prepared in semisolid methylcellulose medium HCC 4230 (Terry Fox Labs, Vancouver,

Canada) or plasma clot (18). Concentrations of growth factors and human AB serum during culture were fivefold lower than those used during the oligodeoxynucleotide treatment. For myeloid colony stimulation (CFU-GM, CFU-M, CFU-Eo, or CFU-G), IL-3, GM-CSF, M-CSF, IL-5, and G-CSF were used, alone or in combination. CFU-E were obtained in the presence of Epo alone. BFU-E were induced in the presence of IL-3 and Epo. CFU-Meg were grown in the presence of IL-6, IL-3, and Epo. Cultures were carried out for 10–12 d, except for CFU-E, cultured for 7–9 d. Plates were then scanned with an inverted microscope and the number of colonies ( $\geq 50$  cells) and clusters (8–40 cells) were counted. Myeloid colonies were identified by visual inspection and erythroid colonies were counted after benzidine staining. Immunofluorescence analysis (23) with a glycoprotein IIb/IIIa-specific mAb (24) identified CFU-megakaryocytic (Meg). Different oligodeoxynucleotide preparations, several bone marrow donors, and the same batches of human AB serum and HCC 4230 medium were used. Results of each experiment represent mean  $\pm$  SD from 8–24 separate colony assays. Student's *t* test was used for statistical analysis.

**Reverse Transcription Polymerase Chain Reaction (RT-PCR).** To analyze the effects of the N-ras oligodeoxynucleotides exposure on N-ras mRNA levels, 10<sup>4</sup> CD34<sup>+</sup> cells were plated into 96-well plates in 0.1 ml of IMDM supplemented with 2% human AB serum, IL-3 and GM-CSF, Hepes buffer, and 80  $\mu$ g/ml of N-ras antisense or sense oligodeoxynucleotides. After 12 h, a second dose of oligomer (40  $\mu$ g/ml) was added and the incubation continued for additional 12 h. Control cells were untreated. Cells were collected separately from each experimental group, centrifuged, washed, and total RNA was extracted as described (20). RNA from each group was divided into two portions. One sample was reverse transcribed using 400 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) and 0.1  $\mu$ g of 3'-end primer of N-ras exon 1 for 1 h at 37°C. The second sample was reverse transcribed using the  $\beta_{2m}$  3' primer. For single colony RT-PCR analysis, individual colonies were plucked from methylcellulose and resuspended in 50  $\mu$ l of IMDM; cells were then centrifuged and lysed for 5 min at 4°C in 7  $\mu$ l of 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 3 mM MgCl<sub>2</sub> and 2% NP-40. The samples were then centrifuged at 12,000 rpm for 2 min at 4°C and the supernatants were used as a source of mRNA for reverse transcription reaction as above. Resulting cDNA fragments were amplified with 5 U of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) in the presence of the 5' primer of either N-ras exon 1 or  $\beta_{2m}$ , generating a 115-bp fragment of N-ras and a 252-bp fragment of  $\beta_{2m}$  cDNA during 50 cycles of PCR (25). Reaction products were subjected to electrophoresis in a 2% agarose gel, transferred to Zeta probe blotting membranes (Bio-Rad Laboratories, Richmond, CA), and hybridized overnight at 55°C using N-ras or  $\beta_{2m}$  synthetic oligomer end-labeled with  $\gamma$ -[<sup>32</sup>P]ATP and polynucleotide kinase as described (26). Filters were washed twice with 2× SSC containing 0.1% SDS at 49°C for 30 min and exposed to x-ray films with intensifying screens. Densitometric measurements of the hybridization bands were performed using an Ultrascan XL (Pharmacia, LKB) apparatus.

## Results

**Effect of N-ras Antisense Oligodeoxynucleotides on Granulocyte/Macrophage Colony Formation.** A-T-MNC were exposed to oligodeoxynucleotides (80  $\mu$ g/ml at time 0; 40  $\mu$ g/ml 18 h later) and plated (5 × 10<sup>4</sup> cells/plate) under optimal culture conditions for the growth of specific progenitor cell subsets.

<sup>1</sup> Abbreviations used in this paper: A-T-MNC, adherent- and T lymphocyte-depleted mononuclear marrow cells;  $\beta_{2m}$ ,  $\beta_2$ -microglobulin; Epo, erythropoietin; Meg, megakaryocytic; RT, reverse transcription.

Treatment with sense oligodeoxynucleotide inhibited colony growth stimulated with IL-3, GM-CSF, M-CSF, G-CSF, or IL-5 by 3.9, 1.5, 15.2, 0.4, and 2%, respectively, compared with untreated controls. Instead, treatment with the *N-ras* antisense oligodeoxynucleotide resulted in 48.7, 36.0, and 67.5% inhibition of colony formation in the presence of IL-3, GM-CSF, or M-CSF, respectively, but did not affect colony formation in the presence of G-CSF or IL-5, compared with sense-treated groups (Table 1). Colony size was only marginally affected by treatment with sense oligodeoxynucleotide, whereas the antisense treatment reduced the size of most colonies growing in the presence of IL-3, GM-CSF, or M-CSF, but not of those growing with IL-5 or G-CSF (data not shown). When A-T-MNC were cultured in the presence of IL-3 + GM-CSF, IL-3 + M-CSF, IL-3 + G-CSF, or IL-3 + IL-5, sense oligodeoxynucleotide did not inhibit colony formation significantly (16.9, 7.4, 0, and 12.1% inhibition, respectively) compared with controls, but treatment with *N-ras* antisense oligodeoxynucleotides resulted in 38.6, 44.8, 35.9, and 38.4% inhibition of colony formation compared with sense-treated cultures (Table 1).

To analyze the effect of *N-ras* antisense oligodeoxynucleotides on the growth of a more homogeneous, less mature population of hematopoietic progenitors, colony assays were performed using CD34<sup>+</sup> cells isolated from A-T-MNC (Table 2). Treatment of the cells with sense oligodeoxynucleotides inhibited colony growth stimulated with IL-3, GM-CSF, M-CSF, or G-CSF by only 0.6, 21.7, 16.7, and 10.7%, respectively, compared with controls, and colony size was not affected. Antisense oligodeoxynucleotide treatment, instead, efficiently inhibited the number of colonies induced with IL-3 (73.6%), GM-CSF (53.3%), and M-CSF (61.5%), as compared with values obtained with sense oligomers (Table 2).

As in the case of A-T-MNC, residual colony formation stimulated by G-CSF was not affected. IL-5 alone was not studied because CD34<sup>+</sup> cells do not form colonies in the presence of this cytokine (27). When IL-3 was combined with GM-CSF, M-CSF, G-CSF, or IL-5, sense oligodeoxynucleotide treatment reduced colony number only slightly (by 10.3, 12.3, 13.3, and 1%, respectively) as compared with controls, whereas antisense treatment significantly inhibited both the size (not shown) and the number of colonies (54.7, 79.1, 59.8, and 37.1%, respectively) as compared with values obtained with the sense oligomer (Table 2).

**Effect of *N-ras* Antisense Oligodeoxynucleotides on Erythroid Colony Formation.** The influence of *N-ras* antisense oligodeoxynucleotides on BFU-E colony formation by A-T-MNC and CD34<sup>+</sup> cells was evaluated in the presence of IL-3 + Epo (Tables 1 and 2). Sense oligodeoxynucleotide treatment did not significantly diminish colony number (10.3 and 11.2% inhibition, respectively) or size compared with controls. The antisense oligodeoxynucleotide inhibited colony number by 20.4% (A-T-MNC) and 40.3% (CD34<sup>+</sup>) compared with the sense oligomer, but only slightly reduced BFU-E colony size (not shown). Neither sense nor antisense oligodeoxynucleotides influenced the number or size of CFU-E colonies from A-T-MNC in the presence of Epo only (Table 1). Colony growth with Epo only was not studied with CD34<sup>+</sup> cells because CFU-E are not contained within this cell population (27).

**Effect of *N-ras* Antisense Oligodeoxynucleotides on Megakaryocytic Colony Formation.** A-T-MNC and CD34<sup>+</sup> cells were incubated in the presence of IL-6 + IL-3 + Epo, and CFU-Meg colonies were identified by mAb immunofluorescence staining. Sense oligodeoxynucleotides did not significantly reduce colony number (8.3 and 14% inhibition com-

**Table 1.** Effect of *N-ras* Oligodeoxynucleotides on In Vitro Colony Formation from A-T-MNC

Inducer	Number of colonies/plate*			Percent inhibition†
	Control	Sense	Antisense	
IL-3	337.0 ± 24.1	325.7 ± 48.3	173.0 ± 39.6	48.7 ( <i>p</i> = 0.035)
GM-CSF	449.0 ± 58.0	465.0 ± 1.4	287.5 ± 30.4	36.0 ( <i>p</i> = 0.016)
M-CSF	75.5 ± 9.2	64.0 ± 4.2	24.5 ± 10.6	67.5 ( <i>p</i> = 0.039)
G-CSF	119.5 ± 34.6	120.5 ± 37.5	133.0 ± 25.5	-10.4 (NS)
IL-5	319.5 ± 46.0	312.8 ± 40.8	323.3 ± 22.1	-3.4 (NS)
EPO	304.0 ± 138.7	284.9 ± 109.6	287.7 ± 135.4	-0.1 (NS)
IL-3 + GM-CSF	431.8 ± 91.5	358.6 ± 49.	213.5 ± 55.3	38.6 ( <i>p</i> < 0.001)
IL-3 + M-CSF	356.5 ± 43.1	330.0 ± 14.1	182.0 ± 5.7	44.8 ( <i>p</i> = 0.005)
IL-3 + G-CSF	355.5 ± 17.7	355.0 ± 43.1	228.0 ± 50.9	35.9 ( <i>p</i> = 0.014)
IL-3 + IL-5	503.5 ± 76.0	423.0 ± 36	260.5 ± 39.6	38.4 ( <i>p</i> < 0.001)
IL-3 + EPO	320.6 ± 61.3	287.5 ± 41.1	228.9 ± 39.6	20.4 ( <i>p</i> = 0.004)
IL-6 + IL-3 + Epo	124.1 ± 22.1	113.8 ± 25.6	20.8 ± 11.1	81.7 ( <i>p</i> < 0.001)

\* Values are mean ± SD from duplicate cultures from four to six separate experiments.

† Inhibition of colony formation by antisense oligodeoxynucleotides in comparison with sense-treated groups (in parentheses, statistical significance).

**Table 2.** Effect of *N-ras* Oligodeoxynucleotides on In Vitro Colony Formation from CD34<sup>+</sup> Cells

Inducer	Number of colonies/plate*			Percent inhibition†
	Control	Sense	Antisense	
IL-3	128.8 ± 27.1	128.0 ± 7.6	34.0 ± 5.8	73.6 (p < 0.001)
GM-CSF	116.2 ± 36.7	91.0 ± 13.6	42.5 ± 2.9	53.3 (p < 0.001)
M-CSF	56.2 ± 14.3	46.8 ± 9.0	18.2 ± 4.3	61.5 (p = 0.001)
G-CSF	16.8 ± 5.2	15.0 ± 3.6	16.0 ± 3.6	-0.1 (NS)
IL-3 + GM-CSF	135.2 ± 17.3	121.3 ± 17.6	54.9 ± 14.2	54.7 (p < 0.001)
IL-3 + M-CSF	172.2 ± 14.2	151.0 ± 24.8	31.5 ± 4.7	79.1 (p < 0.001)
IL-3 + G-CSF	180.4 ± 18.7	156.4 ± 35.9	62.8 ± 23.8	59.8 (p < 0.001)
IL-3 + IL-5	181.0 ± 4.2	202.5 ± 28.3	127.3 ± 22.8	37.1 (p = 0.006)
IL-3 + Epo	187.0 ± 50.8	166.0 ± 27.6	99.1 ± 18.6	40.3 (p < 0.001)
IL-6 + IL-3 + Epo	21.5 ± 3.5	18.5 ± 3.4	6.3 ± 2.5	66.0 (p < 0.001)

\* Values are mean ± SD from duplicate cultures from 6–12 separate experiments.

† Inhibition of colony formation by antisense oligodeoxynucleotides in comparison with sense-treated groups (in parentheses, statistical significance).

pared with controls), whereas antisense treatment caused strong inhibition of colony formation (81.7 and 66% compared with sense) (Tables 1 and 2). Colony size was only slightly reduced in sense-treated cultures but markedly diminished after antisense treatment (Fig. 1).

**Expression of *N-ras* mRNA in CD34<sup>+</sup> Bone Marrow Cells Exposed to *N-ras* Oligomers.** To determine whether the effects of *N-ras* antisense treatment on colony formation correlated with inhibition of *N-ras* expression in hematopoietic cells, CD34<sup>+</sup> cells were exposed to *N-ras* sense or antisense oligodeoxynucleotide and levels of *N-ras* mRNA were analyzed by RT-PCR technique. High levels of *N-ras* mRNA were detected in untreated (Fig. 2, lane *c*) as well as in sense-treated CD34<sup>+</sup> cells (Fig. 2, lane *s*). Significantly lower levels of *N-ras* mRNA were present in cells incubated with antisense oligodeoxynucleotide (Fig. 2, lane *as*). Densitometric measurement of the *N-ras* cDNA hybridizing band in sense-versus-antisense-treated samples indicated that the signal from the antisense-treated cells was <5% that from the sense-treated cells. Levels of  $\beta_2m$  mRNA, used as control, were similar in all samples. Thus, treatment with *N-ras* antisense oligodeoxynucleotides causes a specific downregulation of *N-ras* expression in CD34<sup>+</sup> cells.

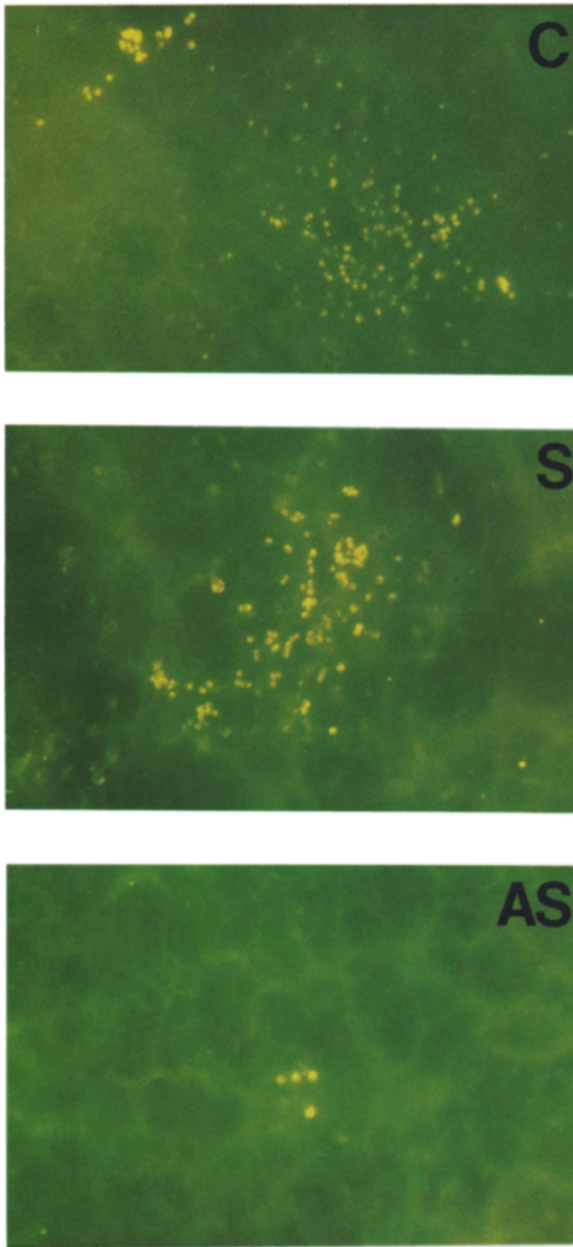
**Detection of *N-ras* Transcripts in Individual Myeloid and Erythroid Colonies.** To determine whether the lack of inhibitory effect of *N-ras* antisense oligodeoxynucleotides on granulocyte and erythroid colony formation was due to the absence of *N-ras* expression in those specific colonies, single colonies obtained after stimulation with GM-CSF, G-CSF, IL-3 + Epo, or Epo were analyzed for *N-ras* mRNA expression. Each CFU-E colony consisted of 40–50 cells, whereas the number of cells in the remaining colonies (BFU-E, CFU-GM, and CFU-G) was 200–250. Detection of *N-ras* mRNA by RT-PCR in each colony type (Fig. 3) indicates that the effect of *N-ras* antisense

oligodeoxynucleotides on the different colonies is not associated with a stage-specific, lineage-dependent pattern of *N-ras* expression.

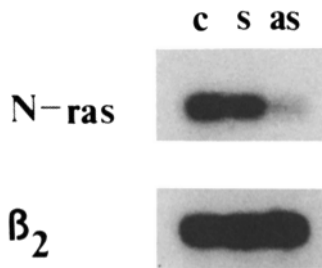
## Discussion

To assess the functional significance of *N-ras* expression in normal hematopoietic progenitor cells, we exposed different progenitor cell subsets to *N-ras* antisense oligodeoxynucleotides and assayed colony formation in the presence of hematopoietic growth factors supporting growth of myeloid, erythroid, or megakaryocytic progenitors. The antisense strategy has been successfully used for the functional analysis of numerous genes (28), including *H-ras* (29). The antisense oligodeoxynucleotide we used is specific for *N-ras*, and does not affect *H-ras* or *K-ras* expression, because of four and three nucleotide mismatches, respectively; two nucleotide mismatches are sufficient to abrogate the biological effect exerted by an 18-mer fully matched antisense oligodeoxynucleotide (30, 31). Our results provide direct evidence that *N-ras* is required for growth of normal human hematopoietic cells. The *N-ras* antisense oligodeoxynucleotide inhibited myeloid colony formation derived from A-T-MNC as well as that derived from CD34<sup>+</sup> cells in the presence of IL-3, GM-CSF, or M-CSF, but not G-CSF or IL-5, except when combined with IL-3. Colony size of most residual colonies formed after *N-ras* antisense treatment in the presence of IL-3, or GM-CSF or M-CSF, was reduced compared with the sense-treated and control groups, although colony morphology was not altered.

The number, but not the size, of early erythroid progenitor colonies (BFU-E) growing from A-T-MNC or CD34<sup>+</sup> cells in presence of IL-3 + Epo was moderately reduced, but no influence of the antisense oligodeoxynucleotide on late

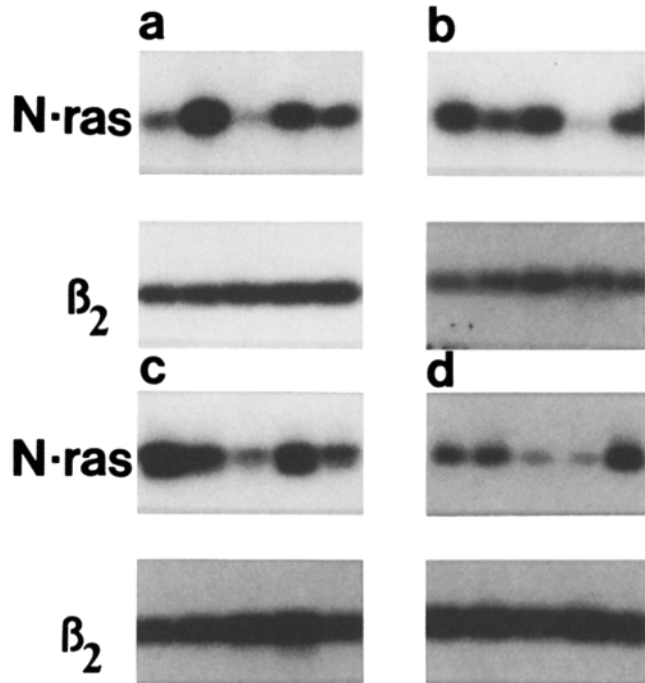


**Figure 1.** Effect of *N-ras* oligomers on size of megakaryocytic colony derived from A-T-MNC in the presence of IL-6 + IL-3 + Epo. (C) control, (S) sense, (AS) antisense.



**Figure 2.** Expression of *N-ras* and  $\beta_2m$  mRNA in CD34<sup>+</sup> cells exposed to *N-ras* oligodeoxynucleotides. CD34<sup>+</sup> cells ( $10^5$ /ml) were left untreated (lane c), or exposed to  $\sim 80$   $\mu\text{g}/\text{ml}$  of *N-ras* sense (lane s) or antisense (lane as) oligodeoxynucleotides, at time 0. After 12 h, a second dose (40  $\mu\text{g}/\text{ml}$ ) was added. Cells were harvested 12 h later, and mRNA was

isolated and divided into two aliquots that were separately amplified with *N-ras* and  $\beta_2m$ -specific primers using RT-PCR. The resulting cDNAs were hybridized to specific  $^{32}\text{P}$  end-labeled probes as described in Materials and Methods.



**Figure 3.** Levels of *N-ras* mRNA in myeloid and erythroid colonies. Five individual erythroid (CFU-E and BFU-E) and myeloid (CFU-GM and CFU-G) colonies were isolated after 5-d (BFU-E), 7-d (CFU-E and CFU-G), and 10-d (CFU-GM) culture in methylcellulose. Cytoplasmic RNA was extracted and RT-PCR performed for detection of *N-ras* transcripts as described in Materials and Methods.  $\beta_2m$  expression served as control. a, BFU-E; b, CFU-GM; c, CFU-G; d, CFU-E colonies.

erythroid progenitor (CFU-E) colony formation was observed. The observation that colony formation was not inhibited in the presence of Epo, G-CSF, or IL-5 except when combined with IL-3 indicates that the lack of antisense inhibitory activity did not depend on either degradation of the oligodeoxynucleotide or inhibition of its cellular uptake by these cytokines. Together, these data suggest that *N-ras* is required for the growth of early but not of late myeloid and erythroid progenitor cells. The observation that the bone marrow CD34<sup>+</sup> subpopulation, which is enriched in pluripotent hematopoietic stem cells, is more sensitive than the A-T-MNC population to the inhibitory effect of the *N-ras* antisense oligodeoxynucleotide is consistent with this hypothesis.

In contrast, during monocytopoiesis, *N-ras* appears to be required by early and late progenitors. Additional evidence to support the interpretation of these findings was obtained using different concentrations of M-CSF to exclude the possibility that high concentrations of the cytokine would stimulate CFU-GM colony formation. Based on morphological criteria, all of the colonies formed in the presence of M-CSF were of monocyte/macrophage origin (data not shown).

*N-ras* is also needed for normal megakaryocytopoiesis, as indicated by the pronounced inhibition of CFU-Meg colony formation after antisense oligodeoxynucleotide treatment.

*N-ras* requirements in normal human hematopoiesis appear to be associated with the activation of specific signal transduction pathways after the interaction of IL-3, GM-CSF,

and M-CSF, but not of G-CSF, IL-5, and Epo, with their respective receptors. Our data using GM-CSF and IL-3 support and extend previous observations on in vitro established cell lines. An increase in GTP-bound p21 levels in cells stimulated with PDGF (11), EGF (12), IL-2, IL-3, and GM-CSF, but not IL-4 (15) or IGF-1 (32), has been reported and, based on that observation, it was suggested that p21 *ras* is required in the signal transduction pathway induced by these growth factors. The human GM-CSF and IL-3 receptors exhibit a striking homology in their primary structure (33); this is likely to explain their crossreactivity with the respective ligands and the apparent use of common molecular mechanisms of signal transduction (34). Accordingly, it might be difficult to determine whether the N-*ras* requirement is specifically associated with the GM-CSF or IL-3-dependent signal transduction pathway.

We report the first evidence, to our knowledge, that N-*ras* is involved in the signal transduction pathway activated by M-CSF. Although there is no demonstration that M-CSF induces an increase in GTP-bound p21 levels directly, transformation of NIH 3T3 cells by the oncogenic form of the M-CSF receptor (*v-fms*) is inhibited by microinjection of the anti-*Ras* p21 antibody Y13-259 (35), supporting our conclusion that N-*ras* activation plays an important role also in the proliferative response induced by activation of the nononcogenic form of the M-CSF receptor.

The possibility that the inhibition of BFU-E we observed upon IL-3 + Epo stimulation does not depend directly on an effect of N-*ras* on erythroid colony formation stimulated by those cytokines cannot be excluded by our data. It has been suggested that PDGF enhances, directly or indirectly, erythroid colony formation induced by Epo (36, 37); the possibility that N-*ras* activation upon Epo stimulation occurs in response to PDGF, released by accessory cells or present in the serum used for culture, is unlikely because N-*ras* antisense oligodeoxynucleotide treatment does not affect CFU-E colonies (Table 1).

The receptors for hematopoietic growth factors either have intrinsic tyrosine kinase activity (M-CSF receptor) or are capable to activate these enzymes (IL-3, GM-CSF, G-CSF, and Epo receptors). Although the activation of the N-*ras* p21 protein after growth factor stimulation might require phosphorylation of the receptors and/or of target molecules, the

mechanisms involved are not understood. A mechanism by which p21 *ras* is maintained in the GTP-bound form involves inactivation of GTPase activating protein (GAP); whether this is affected by phosphorylation or by other mechanisms is not yet clear (13, 15). It is possible, for instance, that the interaction of Epo, or G-CSF or IL-5, with their receptors fails to generate the GTP-bound form of p21 *ras* or that the signal transduction pathway downstream of *Ras* is not activated. Based on our observation that growth factor-induced colony formation has differential requirement for N-*ras*, there appear to be at least two distinct pathways associated with proliferation of hematopoietic progenitor cells: one is associated with IL-3, GM-CSF, and M-CSF stimulation, and the other with G-CSF, IL-5, and Epo activity. Although our findings could be explained on the basis of a lineage-dependent mechanism of N-*ras* activation, two observations are against this interpretation: (a) residual colonies formed in the presence of GM-CSF after exposure to N-*ras* oligodeoxynucleotides were morphologically identified as granulocytic, macrophage, and mixed colonies (not shown); and (b) comparable levels of N-*ras* mRNA were detected in individual erythroid (CFU-E), granulocytic (CFU-G), and granulocyte/macrophage (CFU-GM) colonies (Fig. 3). Further studies are needed to determine the basis for the involvement of N-*ras* in distinct growth factor-dependent signal transduction pathways.

The physiological role of protooncogenes in hematopoiesis most likely involves the coordinate regulation of proliferation and differentiation. Each step in the process of generating terminally differentiated cells from multipotent and unipotent progenitors is probably under the control of several genes encoding products with distinct functions. We have previously demonstrated that *c-myc* (18, 20) and *c-abl* (38) play a regulatory role in normal hematopoiesis, and we now report the involvement of N-*ras* in the process. Our results underscore the complexity of the regulatory mechanisms involved in hematopoiesis and provide a rational basis to explain the pathological effects that may result from aberrant activation of protooncogenes. It seems not unreasonable to hypothesize that the mutated form of N-*ras*, which is detected in many cases of acute myelogenous leukemia, bypasses certain regulatory steps associated with the developmental program of normal hematopoietic cells.

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