Suppression of Telomerase Activity and Cytokine Messenger RNA Levels in Acute Myelogenous Leukemia Cells *in Vivo* in Patients by Amifostine and Interleukin 4[1]

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ABSTRACT

High levels of telomerase activity and high rates of cell proliferation are associated with a poor prognosis in acute myelogenous leukemia. Furthermore, cytokine production by leukemia cells is believed to play an important role in determining the proliferative characteristics of leukemia. The *in vivo* effects of two noncytotoxic agents on these parameters were determined in 33 acute myelogenous leukemia patients. Three daily doses of interleukin (IL) 4 or a single dose of amifostine reduced telomerase activity in the leukemia marrow cells in 7 of 9 and 11 of 13 patients, respectively. The administration of a single dose of amifostine resulted in a reduction in tumor necrosis factor α and IL-6 transcript levels in the marrow cells of 10 of 13 and 12 of 13 patients in which these transcripts were present. The administration of only three doses of IL-4 or a single dose of amifostine has a significant effect on leukemia cell parameters, which are believed to have a significant impact on the *in vivo* biology of the disease and on its response to remission induction therapy.

INTRODUCTION

High proliferative potential and high proliferative rates of leukemic cells contribute to the rate of progression of leukemia and to one type of treatment resistance, regrowth resistance (1, 2). We have previously reported on studies demonstrating that the administration of 13-*cis*-retinoic acid and IFN-α can slow the proliferation of myeloid leukemia cells *in vivo* in patients (3, 4). This study reports that both amifostine and IL[3]-4 can reduce telomerase activity in AML cells *in vivo* in patients and that amifostine can also reduce the TNF-α, IL-6, and increase flt3 transcript levels within AML cells. Given that telomerase activity and cytokine production play a role in determining the proliferative potential (5, 6) and proliferative rate of AML cells (7), the administration of amifostine and/or IL-4 between courses of chemotherapy has the potential to reduce or abrogate regrowth resistance as a cause of treatment failure in AML.

PATIENTS AND METHODS

Patients. A single dose of amifostine was administered to 22 patients. IL-4 was administered to 11 patients daily for 3 days. A diagnosis of AML was confirmed by standard methods including morphological assessment and immunophenotyping. Informed consent was obtained from each patient.

Sample Collection. BM aspirates were obtained from AML patients for study before the administration of IL-4 or amifostine. Patients then received either one dose of amifostine (kindly provided by Alza Corp., Palo Alto, CA) as an i.v. infusion (100 mg/m^2) or three doses of IL-4 (kindly provided by the Schering Plow Corp., Kenilworth, NJ; 2–4 mg/kg^2 daily × 3). Seventy-two h after the amifostine infusion or 24 h after the last dose of IL-4, a second BM aspirate sample was obtained. Part of each BM aspirate was mixed immediately with a 4 M guanidinium thiocyanate solution and processed for assessment of cytokine mRNA levels as described below. Light density mononuclear cells were also obtained for study by Ficoll-Hypaque density gradient centrifugation (specific gravity = 1.077).

Telomerase Activity Assay. Light density mononuclear BM cells obtained by density separation (1.077) were frozen at −130°C. The frozen cells were thawed rapidly in a 37°C water bath. After washing with PBS, protein was extracted for 30 min with cold CHAPS lysis buffer (10^6 cells/200 μl; 0.5% CHAPS, 10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, and 10% glycerol) and centrifuged at 12,000 × *g* for 30 min at 4°C. Each extract (2 μl; equivalent to 5,000 cells) was assayed in a 50-μl reaction mixture.[4]

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[3] The abbreviations used are: IL, interleukin; AML, acute myelogenous leukemia; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; TPG, total product generated; TNF-α, tumor necrosis factor α; BM , bone marrow; RT-PCR, reverse transcription-PCR; FAB, French-American-British; GM-CSF, granulocyte macrophage colony-stimulating factor.

[4] B. Li, S. Bi, J. Yang, C. Andrews, E. Horvath, P. Toofanfard, H. Chopra, X. Z. Gao, E. Devemy, X. K. Huang, J. Cartlidge, A. Raza, and H. D. Preisler. Telomerase activity in human secondary hematologic disorder, submitted for publication.

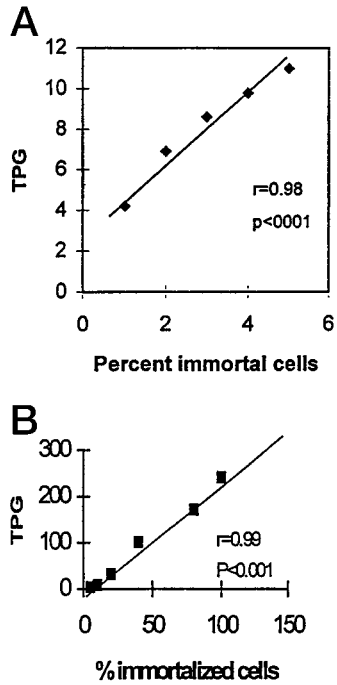

Fig. 1 Reliability of telomerase activity assessment when low and high levels of activity are present. *A*, modification of the standard TRAP assay to permit measurement of low levels of telomerase activity. The TPG number is the arithmetic mean of three tests. *B*, standard curve for the conventional TRAP assay. The TPG number is the arithmetic mean of three tests. Each unit of TPG corresponds to the number of TS primers (in 1×10^3/mole or 600 molecules) extended with at least four telomeric repeats by telomerase in the cell extract. The formula $TPG = (X - X_0/C(C - C_0)/Cr$.

To assay telomerase activity, a semiquantitative approach established by our laboratory was used.[4] Immortal cells, which have high levels of telomerase activity (catalogue number S7700; Oncor, Inc., Gaithersburg, MD), are mixed in different proportions with cells that do not have telomerase activity (RBCs), and a standard telomerase activity curve is generated (100% immortal cells = 5000 cells; 50% dilution of immortal cells = 2500 immortal cells and 2500 RBCs). The telomerase activity in a patient specimen is measured and matched with the corresponding telomerase activity in the standard curve (expressed as the percentage of activity of the pure population of immortal cells). Because we have found that the slope of the curve that reflects telomerase activity is different for cell suspensions containing 0–5% or >5% of immortal cells, the former dilution was used as the standard for assaying low levels of telomerase activity, whereas the latter curve was used to assay higher activity levels (Fig. 1, *A* and *B*).

The telomerase reaction products were amplified in a thermal cycler for 28 rounds at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s in the standard semiquantitative assay,[4] and to measure low levels of telomerase activity, the telomerase reaction products were amplified in a thermal cycler for 35 rounds at 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s in our semimicroquantitative detection assay.[4] The reactions were conducted in the presence of [α^{32}-P]ATP. The reaction product was electrophoresed in 10% polyacrylamide nondenaturing gels and analyzed in a PhosphorImager by ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Assessment of Cytokine Transcript Profiles. To assess the cytokine transcript profiles, the BM aspirate specimens were mixed immediately after removal from the patient with an equal volume of 4 M guanidinium thiocyanate solution to lyse the cells and prevent RNA degradation. Total cellular RNA was extracted according to the guanidinium thiocyanate-phenol chloroform procedure of Chomczynski and Sacchi (8). Extracted RNAs were stored in ethanol at −80°C.

Multiplex RT-PCR. To detect multiple cytokine transcripts simultaneously, we designed a multiplex RT-PCR to assess the transcripts of the following seven cytokine or cytokine-related genes: (*a*) IL-1β; (*b*) TNF-α; (*c*) IL-6; (*d*) GM-CSF; (*e*) IL-1RA; (*f*) SCF; and (*g*) flt3 (9). In the studies described here, β-actin transcripts were used as an internal control. Total cellular RNA was first used as a template to synthesize total cDNA, as described previously (9). The cDNA was then used for further PCR amplification. To reduce possible differences in amplification efficiencies due to the differences in primer lengths and composition, primers were chosen carefully to ensure maximum homology in their composition and annealing kinetics. The amplification condition for each set of primers was established, and the multiplex reaction was achieved by adding each set of primers one at a time. RNA extracted from HL60 cells was used as positive cytokine transcript control.

The sequences of the primers for each cytokine are listed in Table 1. The final reaction conditions for PCR amplification were 3.50 mM $MgCl_2$, 0.32 mM deoxynucleotide triphosphate, and 5 units of Taq polymerase cycled 26 times at 94°C for 50 s, 60°C for 45 s, and 72°C for 45 s, with a final extended elongation at 72°C for 10 min. The PCR-amplified products were then separated on a 1.5% agarose gel (FMC Bioproducts), stained, and photographed. The band densities were measured using EagleSight software (Eagle Eye II; Stratagene). The cytokine and cytokine-related transcript densities were divided by the β-actin transcript density of the same specimen. This value was then used to compare transcript levels in different patient specimens. Normalization of cytokine transcript levels to the β-actin transcript levels permits a reproducible estimate to be made of relative transcript levels independent of the efficiency of the RT-PCR reactions.

Statistical Analyses. Descriptive statistics such as counts, ranges, and percentages were used to present the distribution of the measured parameters. The median and the mean values were used to measure the variability of the parameters. Confidence intervals of parameter means were also generated based on 1 unit of SD. Considering the number of observations that were available in the studies described here, nonparametric statistical techniques were used as the primary analytical methods. Relationships between parameters were determined by the Spearman rank correlation. The significance of changes in parameters between pre- and post-therapy specimens were determined by the Wilcoxon matched-pairs test, which also tests for directional changes. A two-tailed α level of 0.05 was used as the criterion for statistical significance.

Table 1 Sequences of primers used for multiplex RT-PCR detection of cytokines

Flt3(stk-1)	5′ primer: 5′-TCA-AGT-GCT-GTG-CAT-ACA-ATT-CCC-3′	
	3′ primer: 5′-CAC-CTG-TAC-CAT-CTG-TAG-CTG-GCT-3′	208 bp
SCF	5′ primer: 5′-CTG-CTC-CTA-TTT-AAT-CCT-CTC-GTC-3′	
	3′ primer: 5′-CAC-AAG-GTC-ATC-CAC-TAT-ATT-CAC-3′	291 bp
IL-1RA	5′ primer: 5′-ATG-GAA-ATC-TGC-AGA-GGC-CTC-3′	
	3′ primer: 5′-GTC-CTG-CTT-TCT-GTT-CTC-GCT-3′	360 bp
GM-CSF	5′ primer: 5′-ATG-TGG-CTG-CAG-AGC-CTG-CTG-C-3′	
	3′ primer: 5′-CTG-GCT-CCC-AGC-AGT-CAA-AGG-G-3′	424 bp
IL-6	5′ primer: 5′-ATG-AA-C-TCC-TTC-TCC-ACA-AGC-GC-3′	
	3′ primer: 5′-GAA-GAG-CCC-TCA-GGC-TGG-ACT-G-3′	628 bp
TNF-α	5′ primer: 5′-ATG-AGC-ACT-GAA-AGC-ATG-ATC-CGG-3′	
	3′ primer: 5′-GCA-ATG-ATC-CCA-AAG-TAG-ACC-TGC-3′	695 bp
IL-1β	5′ primer: 5′-ATG-GCA-GAA-GTA-CCT-AAG-CTC-GC-3′	
	3′ primer: 5′-CAC-AAA-TTG-CAT-GGT-GAA-GTC-AG-3′	802 bp
β-Actin mRNA	5′ primer: 5′-GGG-TCA-GAA-GGA-TTC-CTA-TG-3′	
	3′ primer: 5′-CTA-GAA-GCA-TTT-GCG-GTG-GAC-3′	1000 bp

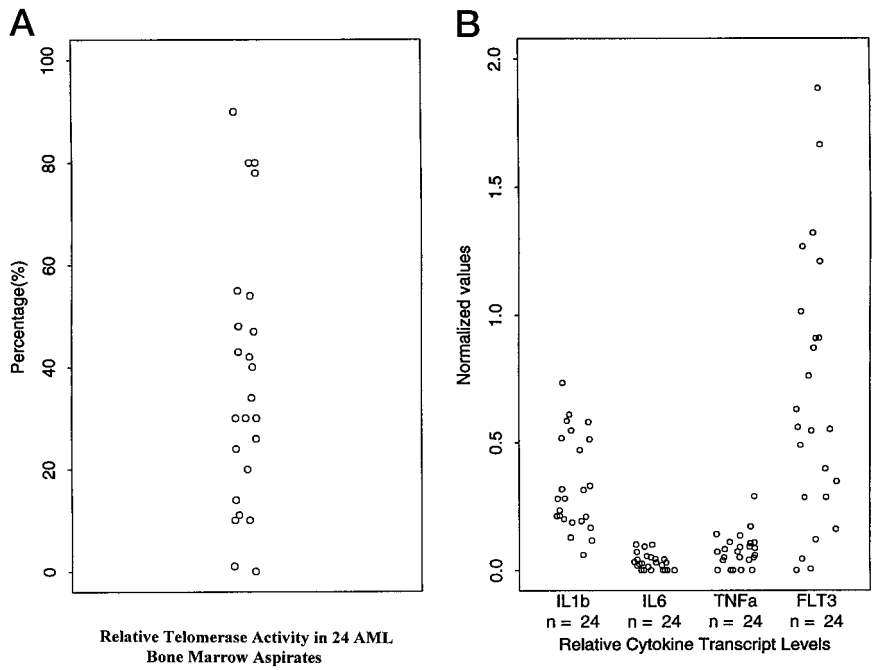

Fig. 2 Telomerase activity and intracellular cytokine transcript levels in AML patients. *A*, telomerase activity as a percentage of 5000 immortal cells. *B*, normalization of transcript levels achieved by dividing the density of the PCR-amplified transcript by the density of β-actin in the same RT-PCR reaction.

RESULTS

General Characteristics of the Patients and Leukemias Studied. The median age of the patients studied was 61 years, with an age range of 20–81 years. There were 15 female and 18 male patients. The FAB classification of the AMLs that were studied are as follows: (*a*) M1, 11 cases; (*b*) M2, 13 cases; (*c*) no FAB M3 cases; (*d*) M4, 5 cases; (*e*) no FAB M5 cases; and (*f*) M6, 1 case. The FAB classification of three of the AMLs was unknown. The median WBC count and platelet counts were 15,600/μl and 49,000/μl, respectively.

Changes in telomerase activity levels were measured in 22 marrow specimens, and changes in cytokine transcript levels were studied in 22 patients. Median and ranges for telomerase activity levels were 32% (0–90%; Fig. 2*A*). Fig. 2*B* provides the ranges of cytokine transcript levels. As we have described previously, AML cells *in vivo* in patients do not contain either GM-CSF or SCF transcripts.

Effects of IL-4 on Telomerase Activity and Cytokine Transcript Levels in Leukemia Cells. Three daily doses of IL-4 were administered to 11 patients. IL-4 administration was associated with a fall in the level of telomerase activity in seven of nine studies from 38.9 ± 11.6% (median, 30%) to 16.7 ± 7.4% (median, 10%; $P = 0.02$; Fig. 3*A*). The effects of IL-4 on cytokine transcript levels within leukemia cells were inconsistent. Changes in telomerase activity were moderately correlated with changes in both IL-1β and TNF-α transcript levels, but these were not significant ($r = 0.42$, $P = 0.30$, and $n = 8$ and $r = 0.32$, $P = 0.44$, and $n = 8$). Changes in IL-1β and TNF-α transcript levels were highly correlated ($r = 0.81$, $P = 0.02$, and $n = 8$; Fig. 3*B*).

Effects of Amifostine on Telomerase Activity and on Cytokine Transcript Levels. A single dose of amifostine was administered to 22 patients. The effect of this agent on telomerase activity was measured in 13 patients, with a fall in activity

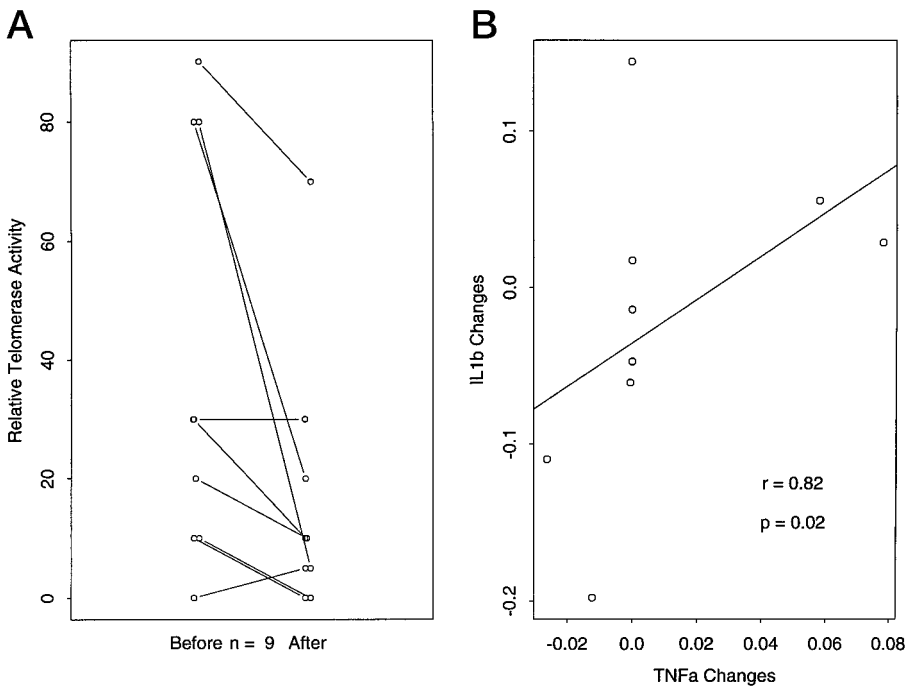

Fig. 3 Effects of IL-4 on AML marrow aspirate cells *in vivo* in patients. *A*, effects on telomerase activity. *B*, relationship between IL-4-induced changes in intracellular IL-1β and TNF-α transcript levels.

occurring in 11 patients (Fig. 4*A*). The mean ± SE and median activity values before amifostine administration were 40% ± 5% (42%), whereas the values after administration were 29.1% ± 3.8% (30%; $P = 0.02$).

In contrast to IL-4, amifostine administration produced significant effects on cellular cytokine transcript levels. A fall in IL-6 transcript levels occurred in 12 of 13 studies ($P = 0.002$; Fig. 4*B*), a fall in TNF-α transcript levels occurred in 10 of 13 studies but lacked significance ($P = 0.21$; Fig. 4*C*), and an increase in flt3 transcript levels was produced in 9 of 13 studies ($P = 0.03$; Fig. 4*D*). Amifostine administration did not produce consistent effects on the other cytokine transcripts that were measured. Whereas there were no apparent relationships between the effects of amifostine and cytokine transcript levels and telomerase activity, there was a correlation between the effects of amifostine on IL-1β and flt3 transcript levels ($r = 0.55$, $P = 0.06$, and $n = 12$; Fig. 4*E*).

DISCUSSION

The studies described here demonstrate that two different noncytotoxic agents have the capacity to reduce telomerase activity within leukemia cells *in vivo* in patients. Amifostine has the further capacity to reduce IL-6, TNF-α, and increase flt3 transcript levels within leukemia cells *in vivo*. The level of telomerase activity is highest in hematopoietic progenitor cells, with the self-renewal capacity of these cells being related directly to the level of telomerase activity (5, 6). Furthermore, high levels of telomerase activity are characteristic of many malignant cell types (10) and are necessary for the immortalization of cells (10). These observations strongly suggest that high levels of telomerase activity in leukemia cells are indicative of and play a role in producing a high self-renewal capacity and proliferative potential in these cells. Given the important role that regrowth resistance plays in the poor responses obtained in a variety of otherwise drug-sensitive malignancies, the administration of either IL-4 or amifostine, by virtue of their effects on telomerase activity, might be expected to reduce the proliferative potential of malignant cells *in vivo*, thereby reducing regrowth resistance. A pilot study of this proposition appears to confirm this conjecture.[5]

Whereas the inhibitory effects of amifostine on cytokine transcript levels do not appear to play a role in the effects of this agent on telomerase activity, these effects are potentially useful. There is evidence of a correlation between the level of TNF-α transcripts and the percentage of S-phase leukemia cells within a leukemia cell population (9).[6] Furthermore, there have been many reports of the stimulatory effects of IL-6 on the proliferation of AML cells (11, 12). Therefore the effects of amifostine on cytokine transcript levels and hence on cytokine production might reduce the number of cells proliferating in an AML marrow as well as their rate of proliferation. Furthermore, the ability of amifostine to reduce

[5] H. D. Preisler, B. Li, P. Venugopal, S. A. Gregory, W-T. Hsu, J. Loew, S. Adler, S. Gezer, R-W. Huang, A. Galvez, D. Slyvnick, R. Larson, and A. Jaje. Poor prognosis AML. I. Response to treatment with high-dose cytarabine/mitoxantrone/amifostine, submitted for publication.

[6] B. Li, J. Nayini, J. Yang, S. Song, S. Perambakam, E. Horvath, M. Tao, Y. Chen, R. Huang, H. Chopra, P. Venugopal, and H. D. Preisler. Poor prognosis AML. II. Biological and molecular biological characteristics, submitted for publication.

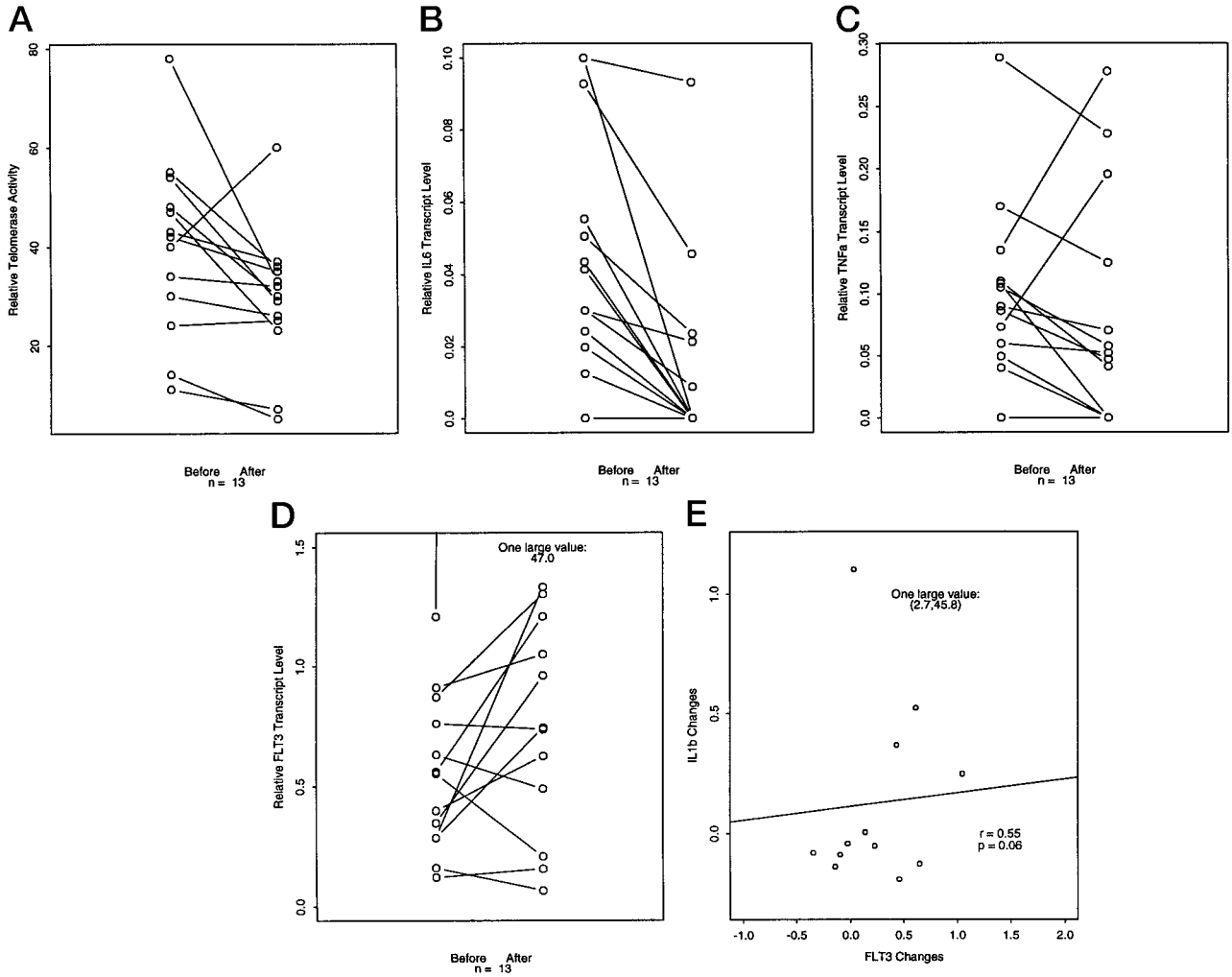

Fig. 4 Effects of amifostine on AML cells *in vivo* in patients. *A*, telomerase activity; *B*, IL-6 transcript levels; *C*, TNF-α transcript levels; *D*, flt3 transcript levels; *E*, association of changes in IL-1β and flt3 transcript levels.

TNF-α transcript levels might explain, at least in part, the reported benefit that this agent produces when used to treat myelodysplasia (13).

Taken together, the above data strongly suggest that the administration of amifostine and/or IL-4 between courses of cytotoxic therapy may play a useful role in improving treatment outcome in AML. A study submitted for publication is consistent with this hypothesis.[5, 6]

REFERENCES

1. Preisler, H. D. Resistance to cytotoxic therapy: a speculative overview. Ann. Oncol., *6:* 651–657, 1995.

2. Preisler, H. D., Raza, A., Bonomi, P., Taylor, S., LaFollette, S., Leslie, W., and Lincoln, S. Regrowth resistance as a likely significant contributor to treatment failure in drug sensitive neoplastic diseases. Cancer Investig., *15:* 358–368, 1997.

3. Preisler, H. D., Raza, A., and Larson, R. A. Alteration of the proliferative rate of acute myelogenous leukemia cells *in vivo* in patients. Blood, *80:* 2600–2604, 1992.

4. Handa, H., Hegde, U. P., Kotelnikov, V., Mundle, S., Dong, L. M., Burke, P., Rose, S., Hsu, W. T., Gaskin, F., Raza, A., and Preisler, H. D. The effects of 13-*cis* retinoic acid and interferon-α in chronic myelogenous leukemia cells *in vivo* in patients. Leuk. Res., *21:* 1087–1096, 1997.

5. Counter, C. M., Gupta, J., Harley, C. B., Leber, B., and Bacchetti, S. Telomerase activity in normal leukocytes and in hematologic malignancies. Blood, *85:* 2315–2320, 1995.

6. Morrison, S. J., Prowse, K. R., Ho, P., and Weissman, I. L. Telomerase activity in hematopoietic cells is associated with self-renewal potential. Immunity, *5:* 207–216, 1996.

7. Bradbury, D., Rogers, S., Reilly, I. A. G., Kozlowski, R., and Russell, N. H. Role of autocrine and paracrine production of granulocyte-macrophage colony-stimulating factor and interleukin-1β in the autonomous growth of acute myeloblastic leukaemia cells—studies using purified CD34-positive cells. Leukemia, *6:* 562–566, 1992.

8. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162:* 156–159, 1987.

9. Gao, X-Z., Bi, S., Chopra, H., Devemy, E., Venugopal, P., Biarou, L., Hsu, W-T., Loew, J., Galvez, A., Gregory, S., Yang, J., Horvath, E.,

and Preisler, H. D. Cytokine gene activity in AML cells *in vivo* in patients. Leuk. Res., *22:* 429–438, 1998.

10. Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L. C., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. Specific association of human telomerase activity with immortal cells and cancer. Science (Washington DC), *261:* 1115–1117, 1994.

11. Oster, W., Cicco, N. A., Klein, H., Hirano, T., Kishimoto, T., Lindemann, A., Mertelsmann, R. H., and Herrmann, F. Participation of the cytokines interleukin 6, tumor necrosis factor-α, and interleukin 1-β secreted by acute myelogenous leukemia blasts in autocrine and paracrine leukemia growth control. J. Clin. Investig., *84:* 451–457, 1989.

12. Beauchemin, V., Villeneuve, L., Rodriguez-Cimadevilla, J. C., Rajotte, D., Kenney, J. S., Clark, S. C., and Hoang, T. Interleukin-6 production by the blast cells of acute myeloblastic leukemia: regulation by endogenous interleukin-1 and biological implications. J. Cell. Physiol., *148:* 353–361, 1991.

13. List, A. F., Brasfield, F., Heaton, R., Glinsmann-Gibson, B., Crook, L., Taetle, R., and Capizzi, R. Stimulation of hematopoiesis by amifostine in patients with myelodysplastic syndrome. Blood, *90:* 3364–3369, 1997.

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