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Antisense Inhibition of c-myc Expression Reveals Common and Distinct Mechanisms of Growth Inhibition by TGFβ and TNFα

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Abstract Downregulation of the c-myc gene in HL-60 cells is associated with growth inhibition and induction of differentiation. Previous studies have reported that the growth inhibitors TGFβ and TNFα downregulate c-myc mRNA levels, suggesting the possibility that these agents may exert some of their phenotypic effects via c-myc downregulation. Our study demonstrates that although both growth inhibitors produce a similar decrease in c-myc protein synthesis, TNFα produces a greater growth inhibition and differentiation induction in HL-60 cells. Combined addition of anti-myc oligomer with either growth inhibitor produces no additive effect. In fact, 4 μM anti-myc oligomer produces the same growth and differentiation effects as does 10 ng/ml TGFβ1. We conclude that downregulation of c-myc expression represents a common mechanism of growth inhibition by TGFβ and TNFα, but that TNFα possesses an additional effect that is independent of c-myc expression.

Key words: leukemia, differentiation, growth factors, HL-60, cell growth

The molecular mechanisms that regulate cell growth and differentiation are believed to involve signal transmission from surface receptors to key nuclear proteins that alter gene expression. There is accumulating evidence that expression of the nuclear proto-oncogenes (c-fos, c-jun, c-myc, c-myb) may be regulated by both stimulatory and inhibitory growth factors, suggesting that alteration in nuclear gene expression might represent a common mechanism of growth factor action [1,2]. A mechanistic role for these nuclear proto-oncogenes is supported by studies that demonstrate that antisense inhibition of these nuclear genes results in growth inhibition and/or induction of differentiation [3–11].

HL-60 promyelocytic leukemia cells are an established model system for analyzing the role of nuclear proto-oncogenes in the regulation of cell growth and differentiation [12,13]. The c-myc gene is amplified five- to 30-fold in the HL-60 cell line and is expressed at high levels [14,15]. Inhibition of c-myc expression represents a common result of differentiation along either the myeloid or monocytic pathway of differentiation [16–20]. In fact, direct inhibition of c-myc expression with antisense RNA results in growth inhibition and induction of differentiation [4–6]. Although the extent and phenotype of differentiation by antisense c-myc varies somewhat in different studies, reports agree that direct inhibition of c-myc expression results in an induction of differentiation. Our prior studies showed induction of nitroblue tetrazolium (NBT) positivity in 20% of anti-myc treated cells [4], whereas Wickstrom et al. reported a greater extent of differentiation induction [6]. Yokoyama and Imamoto have reported that constitutive inhibition of c-myc expression by anti-sense RNA vectors alters the phenotype of HL-60 cells [7]. Their results differ because their transfected cells exhibited predominantly monocytic markers, whereas the anti-myc oligomer-treated cells appeared more granulocytic.

The growth inhibitory agents TGFβ1 *and* TNFα have been shown to inhibit the growth of diverse cell types and induce differentiation in selected settings [21]. This growth inhibition is associated with a downregulation of c-myc gene expression [22–24]. TGFβ1 has been reported to inhibit mouse myelopoiesis [25] and to suppress the growth of human leukemia cells [26,27]. TGFβ1 also increases the relative number of

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granulocyte progenitors during long-term bone marrow culture [28]. TNFα preferentially inhibits the growth of leukemia cells, but does inhibit myeloid progenitor numbers at high doses [29,30]. Studies in HL-60 cells have demonstrated that TNFα inhibits c-myc transcription, which is associated with growth inhibition and the induction of monocytic differentiation [31,32].

In this study, we examined the apparent cause and effect relationship between c-myc downregulation and the effects of inhibitory growth factors. The results suggest that downregulation of c-myc is an important component of the growth inhibitory mechanism for both agents. Downregulation of c-myc may completely account for the effects of TGFβ1, but cannot completely explain the more pronounced effects of TNFα. The results indicate that these growth inhibitors have both common and distinct mechanisms for growth inhibition and differentiation induction.

MATERIALS AND METHODS

Synthesis and Purification of Oligomers

Unmodified 15 base deoxyribonucleotides were synthesized by standard phosphoramidate chemistry and purified by high-pressure liquid chromatography as described previously [4]. The oligomers were characterized by gel electrophorsis on 20% denaturing acrylamide gels following 5′ labeling with polynucleotide kinase. All oligomer experiments included studies with a control sense oligomer to control of non-specific effects. No non-specific effects of control sense oligomer were seen in any of the experiments.

Growth and Differentiation Studies of HL60 Cells

All studies were performed with low passage HL-60 cells analyzed at cell concentration between 200,000 and 1,200,000 per milliliter. All studies employed heat-treated serum to reduce nuclease degradation of oligomers. Pilot studies demonstrated that heat-treatment produced no observable effect on growth inhibition by TGFβ1 or TNFα. Because of the greater extent of anti-myc-mediated differentiation reported by Wickstrom et al. [6], we obtained their HL-60 cell line. Our findings agree with their published report, indicating that the reported differences are true differences between the cell lines. All studies were performed with both the HL-60 cell lines described in our previous studies (HL60N) and the cell line that was graciously provided by Wickstrom and co-workers (HL-60T). Both lines had a low rate of spontaneous differentiation (less than 5% as assayed by NBT positivity), but differed in growth rate. Growth rate and viability were measured by cell counts as described previously [4]. Differentiation was assayed by morphology, induction of nitroblue tetrazolium (NBT) positivity, and cytochemical reactions for napthol AS-D chloroacetate esterase and alpha naphthylacetate esterase were performed as described previously [33]. The percentage of positive cells was assessed by counting 200 cells. Phorbol ester (TPA)-stimulated HL-60 cells were employed as positive controls for the NBT assays. TGFβ1 (R & D systems) and TNFα (Amgen) were diluted appropriately as recommended by the suppliers.

Immunoprecipitation of c-myc Protein

Immunoprecipitation was performed by a modification of the previously described method [34], employing an affinity-purified rabbit polyclonal antibody directed against human c-myc. Cells were incubated with growth inhibitor for 1 to 24 h, washed twice with PBS, and then labeled for 20 min in methionine-free medium (containing 10% heat-treated serum). Sample preparation was performed as described previously, standardized by equal incorporation of trichloroacetic acid precipitable radioactivity.

RESULTS

Growth Inhibitors Reduce c-myc Protein Synthesis

TGFβ1 and TNFα produced a 90% reduction in the synthesis of both of the major c-myc proteins (Fig. 1). Densitometric analysis demonstrated an approximately equivalent decrement in the predominant p64 (ATG initiation) and in the p67 proteins (CTG initiation). The two forms of c-myc protein have been previously described and shown to result from different initiation sites [34]. TGFβ1 and TNFα produced similar inhibitions of c-myc protein synthesis in three separate experiments. The extent of inhibition was similar to that which we previously observed with anti-myc oligomers [4]. One question that arose from our prior anti-myc experiments was whether the incomplete differentiation observed was the result of an incomplete inhibition of c-myc expression or from a requirement for other events to assure complete differ-

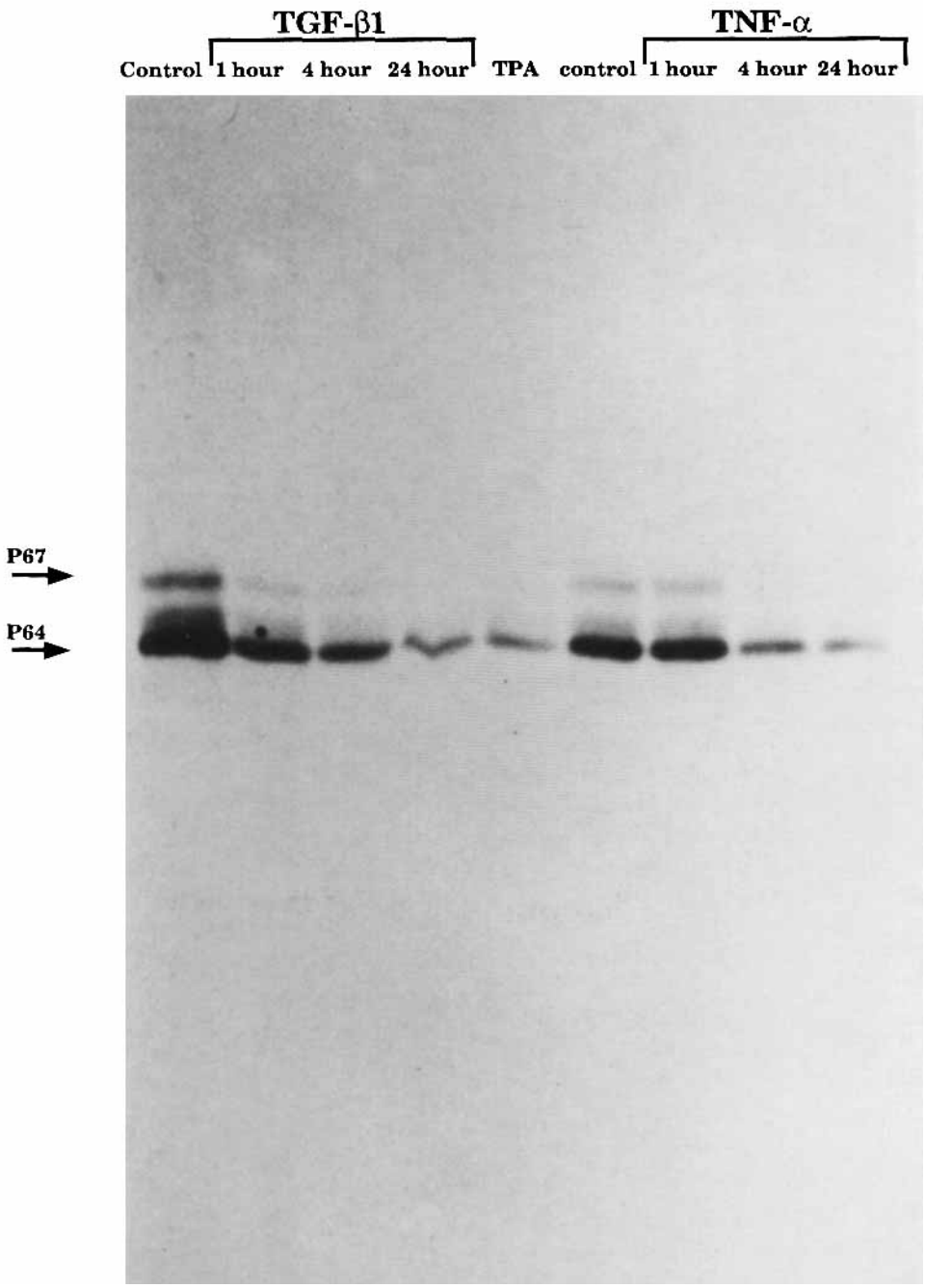

Fig. 1. Autoradiograph demonstrating the rate of c-myc protein synthesis. Equal numbers of trichloroacetic acid-precipitable counts were employed for immunoprecipitation with the affinity purified rabbit polyclonal antibody. Cells were pulse labeled for 20 min following incubation with the stated growth factor. TPA-treated cells were grown in 10 nM TPA for 24 h prior to labeling; control cells were also grown for 24 h prior in standard media prior to labeling with ^{35}S-methionine. Simultaneous growth and differentiation assays were performed to correlate the extent of c-myc downregulation with phenotypic effects. The experiment was repeated three times to confirm these results.

entiation in all of the cells. In order to resolve this question, we compared the effects of three agents that produced approximately equivalent decrements in c-myc protein synthesis: anti-myc oligomer, TGFβ1, and TNFα.

The observed decrease in c-myc protein synthesis was regulated at the transcriptional level. S1 nuclease protection assays demonstrated that the decreased c-myc expression was the result of decreased levels of c-myc mRNA following addi-

tion of TGFβ1 or TNFα (data not shown), which correlated with nuclear run on transcription assays showing that these agents produced decreased transcription of the c-myc gene (data not shown). The extent of transcriptional inhibition by these factors roughly correlated to the extent of inhibition of c-myc protein synthesis shown in Figure 1.

TGFβ1 Inhibits HL-60 Cell Growth

Because TGFβ1 inhibited c-myc protein synthesis, it was important to determine whether this factor would inhibit growth and induce differentiation of HL-60 cells (note that a prediction of our previous work [4] is that agents that decrease c-myc expression should produce growth inhibition and differentiation of HL-60 cells). Figure 2 demonstrates that TGFβ1 produces a dose-dependent inhibition of cell growth in both of the HL-60 cell lines that we evaluated. Because TGFβ1 was diluted in an acidic vehicle, we added equivalent amounts of the vehicle and observed no effects on growth or differentiation.

Non-Additive Effects of TGFβ1 and Anti-myc Oligomers on Growth

Although TGFβ1 decreased c-myc protein synthesis in HL-60 cells (Fig. 1) and induced differentiation (Fig. 2), it had not been proven that this decrease is responsible for its growth inhibitory and differentiating effects. To test the hypothesis that TGFβ1-mediated inhibition of c-myc protein synthesis was responsible for its phenotypic effects, we incubated TGFβ1 and anti-myc oligomers and analyzed the consequent effects on growth and differentiation. We reasoned that if TGFβ1 and anti-myc oligomers produced their effects through the same molecular mechanism (inhibition of c-myc protein synthesis), then their combined effects might be non-additive. Figure 3 demonstrates that the addition of both TGFβ1 (10 ng/ml) and anti-myc oligomer (4 μM) results in a non-additive inhibition of cell growth. Comparison of Figures 3A and 3B shows that TGFβ1 has a greater inhibitory effect on HL-60T cells than on HL-60N cells (62 ± 0.9% and 54 ± 0.7%, respectively). Concentrations of TGFβ1 that gave submaximal growth inhibition produced less growth inhibition than did 4 μM anti-myc oligomers, but still showed no additive effect (data not shown). Addition of sense oligomers produced no significant inhibition of cell growth or induction of differentiation, as reported previously [4].

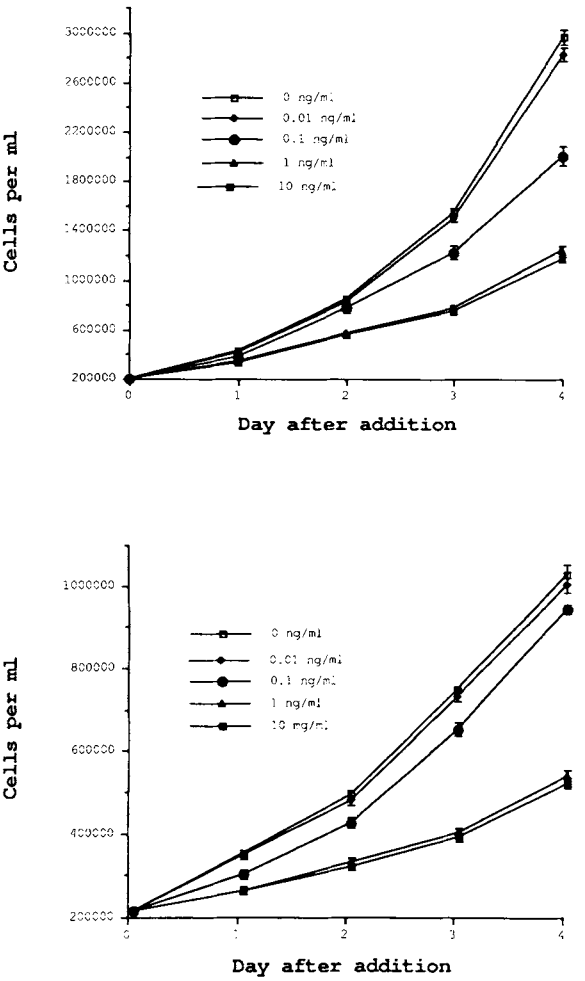

Fig. 2. Growth curves of HL-60 cells incubated with varying concentrations of TGFβ1. Points and bars represent the mean of eight samples, plus and minus standard errors. The standard errors for many points cannot be seen because of the relatively small variation in the experiments. The symbols that represent varying concentrations of TGFβ1 are presented in the legend shown within the graph. Results represent pooled data from two experiments, with four replicates for each point. Cells were diluted with fresh media at a concentration of 1 million cells per milliliter to control for saturation effects, as described previously. Note that the origin of the y axis is at 200,000 cells per milliliter, the concentration of the cells at the time of growth factor addition in all experiments. The **upper panel** shows the results of studies on HL-60N, while the **lower panel** shows results of studies on HL-60T.

TNFα Produces Greater Growth Inhibition Than Anti-myc Oligomers

Previous investigators have reported that TNFα produces decreased c-myc expression and produced differentiation of HL-60 cells. These studies show that TNFα produces a greater growth inhibition than do either TGFβ1 or anti-myc oligomers (compare Fig. 3 with Fig. 4). This

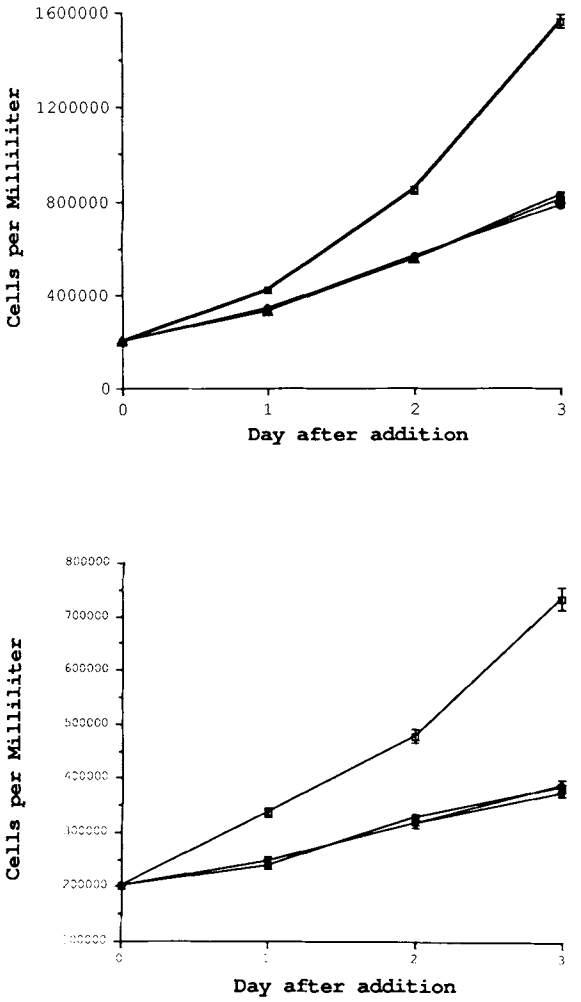

Fig. 3. Growth curves of HL-60 cells incubated with 1 ng/ml of TGFβ1 and/or 4 μM anti-myc oligomer. Points and bars represent the mean of eight samples, plus and minus standard errors. The standard errors for many points cannot be seen because of the relatively small variation in the experiments. The open squares mark the cells treated only with sense oligomer. The symbols for TGFβ1 and/or anti-myc oligomer treatment are difficult to distinguish because the lines are nearly superimposable. Results represent pooled data from two experiments, with four replicates for each point. The **upper panel** shows the results of studies on HL-60N, while the **lower panel** shows results of studies on HL-60T.

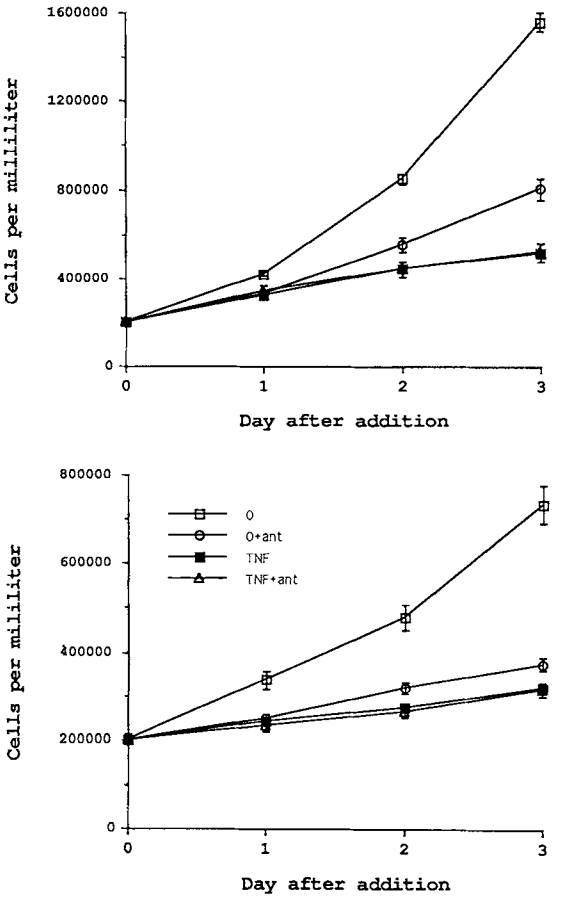

Fig. 4. Growth curves of HL-60 cells incubated with 10 ng/ml of TNFα and/or 4 μM anti-myc oligomer. Points and bars represent the mean of eight samples, plus and minus standard errors. The standard errors for many points cannot be seen because of the relatively small variation in the experiments. The open squares mark the cells treated only with sense oligomer. The symbols for TNFα treatment alone and for TNFα treatment combined with anti-myc oligomer are difficult to distinguish because the lines are nearly superimposable. Results represent pooled data from two experiments, with four replicates for each point. The **upper panel** shows the results of studies on HL-60N, while the **lower panel** shows results of studies on HL-60T.

occurred in both of the HL-60 cell lines tested. TNFα produced a 72 ± 0.6% inhibition in HL60T and a 68 ± 0.7% inhibition in HL60N. However, the combined effects of TNFα and anti-myc oligomer produced a non-additive inhibition of both cell lines. This lack of an additive effect on growth inhibition was not the result of a maximal growth inhibition, because 1.25% dimethylsulfoxide (DMSO) and TPA both produced a greater inhibition of cell growth than did TNFα.

TGFβ1 and TNFα Induce Differentiation That Is Not Increased by Addition of Anti-myc Oligomer

TNFα produced a greater induction of differentiation than did either anti-myc oligomer or TGFβ1 (Fig. 5). Addition of anti-myc oligomer produced no further increase in the percent of differentiating cells, but the induction of differentiation by TNFα was greater than by anti-myc oligomers alone (despite equivalent decreases in levels of c-myc protein). In contrast, differentiation induction by TGFβ1 and anti-myc oligomers was nearly identical and showed no additive effect (Fig. 5). The differences between TNFα

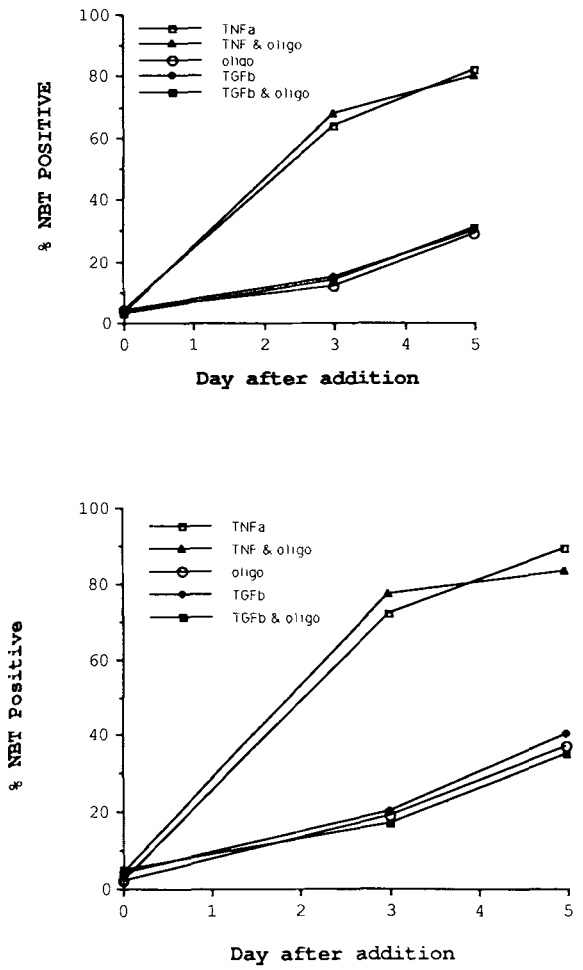

Fig. 5. Graph illustrating the degree of differentiation of HL-60 cells treated with different agents, assayed by the percent of cells that reduce nitroblue tetrazolium (NBT). The symbols for various treatments are shown in the legend within the graph. The concentrations of each agent were the following: TNFα 10 ng/ml, TGFβ1 1 ng/ml; anti-myc oligomer 4 μM (20 μg/ml of oligonucleotide). The **upper panel** shows the results of studies on HL-60N, while the **lower panel** shows results of studies on HL-60T. This experiment was repeated three times and confirmed these results. Control cells never had greater than 3% NBT positivity in the absence of inducing agents, as we have published previously [4].

and TGFβ (or anti-myc oligomer) were most striking in differentiation assays, as TNFα induced two to three times more NBT positive cells.

DISCUSSION

In this work we showed that TGFβ1 and TNFα inhibit c-myc protein synthesis in HL-60 cells and produce differing degrees of growth inhibition and induction of differentiation. These phenotypic differences occur despite similar levels of inhibition of c-myc protein synthesis. Combined addition of anti-myc oligomer with either growth inhibitor results in no additive effect on cell growth or differentiation. These studies are not consistent with the hypothesis that the regulation of c-myc expression completely explains HL-60 growth and differentiation and suggest that TGFβ1 and TNFα employ both common and distinct mechanisms of action.

These studies demonstrate that TGFβ1 and TNFα produce an inhibition of c-myc protein synthesis in HL-60 cells (Fig. 1). Levels of c-myc protein synthesis are also decreased to a similar extent in BALB-MK (mouse keratinocyte) cells treated with TGFβ1 (Pietenpol, Lyons, Holt, and Moses, manuscript in preparation). Comparison of the TGFβ1 doses that inhibit HL-60 cell growth in this study agree with prior studies of MK cells, in which growth inhibition is detectable at 0.1 ng/ml but maximal at concentration between 1 and 10 ng/ml.

TGFβ1 and TNFα produced different degrees of growth inhibition and differentiation induction despite similar decrements in c-myc protein synthesis. Previous studies have reported that TNFα produces monocytic differentiation of HL-60 cells [29], which is consistent with the morphologic appearance and differentiation marker results that we obtained (data not shown). TGFβ1 has been reported to produce a more granulocytic differentiation pattern in leukemia cells [28]. However, because both the monocytic and granulocytic differentiation pathways differ in important ways from normal hematopoietic differentiation, we have chosen to concentrate on differences in the extent of growth inhibition and differentiation rather than to speculate on whether different pathways are involved. Although both inhibitory agents produced similar decrements in c-myc expression, we could not assess the functional significance of this decrement by analyzing levels alone. Either of these agents could have employed a "myc-independent" pathway for growth inhibition or differentiation induction, despite decreasing levels of c-myc mRNA. We have previously reported in collaborative studies that c-fos-dependent and -independent pathways are involved in induction of gene expression [35]. An antisense approach was employed in the present study to determine the extent of the requirement for inhibition of c-myc protein during both TGFβ1- and TNFα-induced differentiation.

Anti-myc oligomer produced different extents of differentiation induction in two different HL-60 cell lines, allowing a controlled analysis of the relationship between c-myc gene inhibition and the effects of TGFβ1 and TNFα. These results indicate that the greater phenotypic effects of anti-myc oligomers reported by Wickstrom et al. [6] result from differences in the cell line. This HL-60T cell line had a greater response to both anti-myc oligomers and TGFβ1 in multiple experiments (compare Fig. 2A to 2B; and Fig. 5A to 5B). The strong similarities between the effects of TGFβ1 and those of anti-myc oligomers were observed in both of the HL-60N and HL-60T cell lines, supporting the idea that the growth inhibitory effects of TGFβ1 may be mediated through inhibition of c-myc expression. We cannot explain the differences between our studies and those of Debenedetti et al. [36] that reported synergism between TGFβ and TNFα (although TNFα did produce greater differentiation induction); addition of both factors did not produce even an additive effect in either of the cell lines employed in our studies.

TNFα produced a greater growth inhibition and induction of differentiation than did either TGFβ1 or anti-myc oligomer. This suggests that part of the phenotypic effects of TNFα are c-myc independent and are distinct from molecular mechanisms employed by TGFβ1. Although this work did not further characterize this c-myc independent pathway, it has been reported that TNFa induces c-fos and c-jun in a variety of cell lines. We have observed induction of c-fos and c-jun transcription in HL-60 cells by TNFα, but not by TGFβ1 (Salhany and Holt, unpublished data). Further studies are necessary to establish the functional significance of TNFa induction of these genes.

In this study, we employed a new approach: the combined use of agents with antisense oligomers directed against agent-regulated genes in order to determine the significance of specific alterations in gene expression. This may represent a general strategy for establishing the difference between pertinent events and non-essential effects on gene expression with an important caveat: Gene inhibition by the antisense oligomers must produce the maximal necessary effect to produce the observed phenotype. If the gene inhibition was incomplete, or small differences in expression levels produced dramatic effects, then this approach would not produce clear-cut results.

The possible use of antisense gene therapy as a treatment for viral diseases and cancer is frequently discussed, despite obvious problems and limitations of this approach [37]. A major drawback of antisense oligomer treatment of cells is the incomplete inhibition of the leukemic population, as more than 20% of the cells usually continue to grow. The use of combination chemotherapy has been proposed, but previous work has shown that some chemotherapeutic agents downregulate c-myc and this study indicates that differentiating agents such as TGFβ1 and TNFα exert at least part of their effect via c-myc downregulation. These findings indicate that anti-myc oligomers may not produce additive inhibition with conventional growth suppressive therapy, because downregulation of c-myc represents an important biologic mechanism for growth inhibition that is shared by many agents and growth inhibitors.

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