Inhibition of c-fes Expression by an Antisense Oligomer Causes Apoptosis of HL60 Cells Induced to Granulocytic Differentiation

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Summary

The c-fes protooncogene is expressed at high levels in the terminal stages of granulocytic differentiation, but so far no definite function has been attributed to the product of this oncogene. To tackle this problem, the c-fes protooncogene expression has been inhibited in HL60 cells, and fresh leukemic promyelocytes of acute promyelocytic leukemia have been induced to differentiate with retinoic acid (RA) and dimethylsulfoxide (DMSO). Inhibition was obtained by incubating the cells with a specific c-fes antisense oligodeoxynucleotide. It was observed that the cells, rather than differentiating, underwent premature cell death showing the morphological and molecular characteristics of apoptosis. This process was inhibited by granulocyte and granulocyte/macrophage colony-stimulating factor, but not by interleukin 3 (IL-3), IL-6, or stem cell factor. Our present results demonstrate that the loss of cell viability that occurs during the in vitro differentiation of myeloid cells, after the complete inhibition of the c-fes gene product and treatment with RA-DMSO, is due to activation of programmed cell death. It is concluded that a possible role of the c-fes gene product is to exert an antiapoptotic effect during granulocytic differentiation.

The c-fes protooncogene is the cellular equivalent of the v-fes, the transforming oncogene of the Snyder-Theilen feline sarcoma virus (1, 2). This gene has been mapped on chromosome 15q25-26 (3) and the complete sequence of the genetic locus has been reported (4–6). The gene codes for a tyrosine kinase protein, the $p92^{c-fes}$ (7), that forms, together with the c-abl product, the family of the cytoplasmic tyrosine kinases (8). The 5' region of this gene is unique when compared with the other tyrosine kinases of the src and receptors gene family (9, 8). In spite of its localization near the breakpoint of the t(15;17), characteristic of the acute promyelocytic leukemia (APL),[1] the c-fes oncogene is not involved in this specific translocation (10, 11). Previous studies in our laboratories did not show any alteration of the c-fes gene locus in the HL60 myeloid cell line as well, which has

been accurately screened before our expression studies. These studies have shown that the c-fes mRNA is detectable at a considerable level in myeloid proliferating cells, and that the level of expression increases during the terminal differentiation to granulocytes and, though at lower level, to monocytes (12–14). Other studies have shown that the c-fes gene product, the $p92^{c-fes}$ protein, is detectable only in myeloid cells (15–18). To tackle the problem of the specific function of this gene in myeloid differentiation, we have inhibited the expression of the c-fes protooncogene in HL60 cells induced to differentiate with retinoic acid (RA) and DMSO, using the methodology of antisense oligodeoxynucleotides (19, 20). Our results showed that HL60 cells treated for 5 d with c-fes antisense (AS) oligomer and induced to differentiate with RA and DMSO undergo to progressive cell death (21). Rather than reaching the morphology of terminally differentiated granulocytic cells, the AS-treated cells retain their immature phenotype and begin to die after 15 h of treatment with the granulocytic differentiation inducers. After 4 d of this treatment, >90% of HL60 cells are dead, showing pycnosis and karyorrhexis. These morphological aspects suggest that the

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[1] Abbreviations used in this paper: APL, acute promyelocytic leukemia; AS, antisense; DP, direct primer; RA, retinoic acid; RP, reverse primer; S, sense; SCF, stem cell factor; Vit. D3, 1a, 25-dihydroxyvitamin D3.

observed events are the expression of an apoptotic process rather than the consequences of a toxic effect of the AS treatment (22, 23). In fact, the same c-fes AS oligomer does not have any toxic effect on the HL60 cells induced to differentiate to monocytes or macrophages by 1a, 25-dihydroxyvitamin D3 (Vit. D3) or tumor-promoting activity (TPA) (21). A set of experiments was therefore planned to characterize this cell death. Besides the morphology of the cells, apoptosis is characterized by a typical pattern of cleavage of DNA into integer multiples of ~200-bp fragments, a length roughly corresponding to the amount of DNA in one nucleosome unit (24). Furthermore, apoptosis has been found to be inhibited by several survival factors (25), which in the hemopoietic system are mainly growth factors such as IL-3, GM-CSF, IL-6, and G-CSF (26). The dependence of the apoptotic process on protein synthesis has been shown in HL60 cells, where cycloheximide induces large scale apoptosis (27). Our results indicate that both in HL60 cells and in freshly collected leukemic promyelocytes the inhibition of c-fes expression after induction to granulocytic differentiation leads to the activation of nuclear events underlying programmed cell death.

Materials and Methods

Cell Cultures. HL60 cells were cultured in 24-well tissue culture plates. Each well contained 7×10^5 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine. Highly homogeneous leukemic blast cells, obtained by bone marrow biopsy of a patient with APL, M3 phenotype carrying the t(15;17), were cultured in the same medium. Differentiation was induced by addition of 1 μM RA plus 1% DMSO. In these culture conditions, terminal differentiation was obtained after 5 d (21). Morphology was assayed by cytocentrifugation followed by May Grunwald Giemsa (MGG) staining. Cytochemical reactions for naphthol-AS-D-chloroacetate esterase, a-naphthyl acetate esterase, and myeloperoxidase were performed by standards methods (28, 29). Purified peripheral blood granulocytes or mononuclear cells were used as positive controls for cytochemical assays.

Synthesis and Purification of Oligomers. Nonmodified deoxyribonucleotides were synthesized on an automated solid-phase synthesizer (381A; Applied Biosystems Inc.) by standard b-cyanoethyl-phosphoramidite chemistry, extracted several times with ammonium-hydroxide and ethanol precipitated as described (30). All oligomers were lyophilized and suspended directly in cell culture medium. The sequences of synthesized oligomers and their relationship to the organization of c-fes coding regions (4, 5) are shown in Table 1. Homology between the oligomers and the human sequences present in the GenBank data bank was no greater than 70%. Furthermore, the 5' region of the c-fes protooncogene, where the AS oligonucleotide was selected, can be considered unique (1, 5). In fact, comparison with sequences of the genes encoding several protein kinases, particularly the tyrosine kinases belonging to the src family, revealed a low degree of homology (8, 9).

Determination of Oligodeoxynucleotides Stability. All the experiments were performed with a single lot of FCS that had been inactivated at 65°C for 20 min, and selected for minimal nuclease activity. Low specific radioactivity ^{32}P end-labeled (31) oligomers were added to the medium at a final concentration of 10 μM and incubated for 24, 48, 72, and 96 h at 37°C. Integrity of the oligomers was evaluated by PAGE. We selected a serum in which the oligodeoxynucleotides were stable for >24 h.

Delivery System of the c-fes Oligomers into Proliferating HL60 Cells. HL60 and fresh leukemic blast cells express high levels of transferrin receptor. We used in our experiments a delivery system based on receptor-mediated endocytosis to introduce the c-fes oligomers complexed with transferrin-polylysine conjugate into HL60 cells as described (32). With this delivery system the oligomer uptake efficiency was, in our hands, ~10 times higher as compared with noncomplexed oligomers. The uptake efficĩency was evaluated as described (33). The DNA/RNA duplex resistant to S1 nuclease digestion was detected 4 and 24 h after culture of HL60 cells in the presence of labeled c-fes AS oligomer-transferrin-polylysine complex as described (21).

Oligomer Treatment of the Cells. c-fes sense (S) or AS oligomers were added to the cultures every 24 h to maintain a final concentration of ~10–15 μM. Control cultures were left untreated. After 3 d of oligomer treatment the culture medium was replaced with fresh medium, containing 10 μM c-fes S or AS oligomer. After 120 h of oligomer treatment RA-DMSO was added to the cultures at the indicated concentration. The experimental conditions and incubation period for these inducers are detailed elsewhere (34, 35). Recombinant human growth factors were added to different cell cultures after the inactivation of the c-fes gene product at the following concentrations: IL-3 (5 ng/ml; Genzyme Corp., Cambridge, MA), IL-6 (10 U/ml; Sigma Chemical Co., St. Louis, MO), stem cell factor (SCF) (10 ng/ml; Genzyme Corp.), GM-CSF (20 ng/ml; Genzyme Corp.), G-CSF (1,000 U/ml; British Biotechnology Ltd., Cowley, Oxford, UK). The cytokines were added simultaneously with the differentiation inducers and in the presence of 10 μM of c-fes S or AS oligomers.

Detection of c-fes mRNA in HL60 Cells Untreated and Treated with c-fes or AS Oligomer. Total cellular RNA was extracted from 7×10^5 HL60 cells exposed to c-fes oligomers for 120 h and from untreated cells, in the presence of 10 μg of Escherichia coli tRNA, using a modification of the guanidinium isothiocyanate procedure (36). RNA was then reverse transcribed using 400 U of murine Moloney leukemia virus reverse transcriptase (MuLV RT; BRL Life Technologies Inc., Gaithersburg, MD) and 0.5 μg of c-fes reverse primer (RP) oligomer for 1 h at 37°C in 1× RT-PCR buffer (10 mM Tris, pH 8.3, 50 mM KCl, 1 mM MgCl$_2$, 10 mM DTT, 200 μM dATP, dCTP, dGTP, dTTP). The oligomers used in the RT-PCR reaction are reported in Table 1. The resulting specific cDNA fragments were amplified with 3 U of Taq polymerase (Promega Biotec, Madison, WI) in the presence of 0.5 μg of c-fes direct primer (DP) oligomer in 1× RT-PCR buffer. Thus, a 258-bp fragment corresponding to the c-fes coding region, nucleotides (nt) 163–574 of the genetic locus, was generated during 35 cycles of PCR (94°C for 40 s, annealing at 50°C for 2 min, and at 72°C for 4 min). 50 μl of the 100 μl PCR volume was separated on a 4% Nusieve (FMC-Bio Products, Rockland, ME) agarose gel and transferred by the electroblotting procedure (37) to a Gene Screen membrane (NEN-DuPont, Boston, MA). The synthetic oligomer probe was end-labeled with γ-[^{32}P]ATP by polynucleotide kinase as described (31). Blots were hybridized with a c-fes oligomer probe, using the experimental condition described by Albretsen et al. (38). As a control for the amount of RNA used as substrate, oligomers specific for the β_2-microglobulin cDNA (39) were used, in a parallel amplification reaction.

Determination of Growth Rate and DNA Content of HL60 Cells after 120 h of Oligomer Treatment. The HL60 cell line has a doubling time of 23 h and a low rate of spontaneous differentiation (<5%). The DNA content of untreated and oligomer-treated cells was determined by flow cytometry according to Dolbeare et al. (40) using a FACScan® (Becton Dickinson & Co., Mountain View,

CA) equipped with a single 488-nM argon laser. Briefly, 10^6 cells were incubated in cell culture medium with 10 μM bromodeoxy-uridine (BrdU) (Sigma Chemical Co.) at 37°C for 30 min. A mouse mAb against BrdU (anti-BrdU; Becton Dickinson & Co.) was bound to BrdU incorporated into neosynthesized DNA. The complex was detected by a rabbit fluorescent anti-FC antibody (Dako Patts A/S, Glostrup, Denmark). DNA histograms were generated from 70% ethanol-fixed and RNase-treated (1 mg/ml) cells. Cells were stained with propidium iodide (PI) (50 μg/ml) and analyzed using the DNA cell cycle analysis program (C-12/86). Every 24 h, for 5 d, at least 10^4 cells were subjected to replicate analysis with <5% standard error. Viability, determined by the trypan blue exclusion test, in untreated and oligomer-treated cultures after 5 d of growth was 90–95%. Experiments were repeated several times.

Measure of HL60 Apoptosis by PI Staining and Flow Cytometry. The 200 g centrifuged pellet (5 × 10^5 cells) was suspended in 500 μl of hypotonic fluorochrome solution (50 μg/ml PI, 0.1% sodium citrate, 0.1% Triton X-100). The samples were placed at 4°C in the dark at least 30 min before the flow cytometric analysis (41), performed with a FACScan® (Becton Dickinson & Co.).

DNA Extraction and Analysis of DNA Fragmentation. DNA extraction was performed using a modification of the technique described by Gross-Bellard et al. (42). Briefly, the cells were pelleted and washed twice in PBS. The pellet was suspended in 0.4 μl of SEDTA (0.1 M NaCl, 50 mM Na$_2$EDTA, pH 7.8) containing 33 μl of 10 mg/ml proteinase K and 40 μl of 10% SDS. The lysate was incubated for at least 4 h at 37°C, then extracted once with 1 vol of SEDTA saturated phenol and 1 vol of CIA (2% isoamyl alcohol in chloroform). The supernatant was precipitated overnight at -20°C with 40 μl of 3 M Na acetate, pH 5, 15 μg of *E. coli* tRNA, and 2.5 vol of cold 100% ethanol. The pellet was suspended in 0.4 ml of 1× TE buffer (10 mM Tris, 1 mM Na$_2$EDTA, pH 8), then 1 μl of 10 mg/ml DNase-free RNAse was added, and the suspension was incubated for 15 min at 37°C. A second digestion with proteinase K was carried out for 1 h at 37°C with 100 μl of proteinase K in SEDTA (1 ml of 10 mg/ml proteinase K and 9 ml of SEDTA) after addition of 10 μl of 10% SDS. The DNA suspension was then extracted once with 0.5 ml of 1:1 phenol/chloroform isoamyl alcohol (CIA), and the supernatant was precipitated as before. The pellet, after washing with 70% ethanol, was vacuum dried and suspended in 50 μl of 1× TE buffer. DNA samples were then separated on 1.2% agarose gel in TBE buffer (90 mM Tris borate, 90 mM boric acid, 2 mM EDTA) containing 1 mg/ml ethidium bromide.
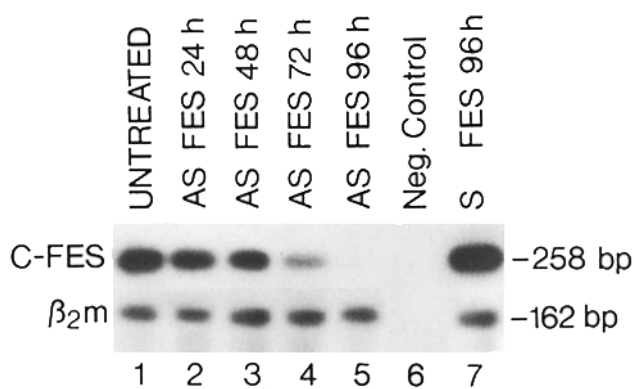

Figure 1. Detection by RT-PCR of the *c-fes* mRNA in HL60 cells treated with the *c-fes* AS oligomer for the time indicated. Total cellular RNA was extracted every 24 h for a period of 4 d after *c-fes* AS oligomer treatment as described in Materials and Methods. The 258-bp amplified fragment was obtained using the DP1 sequence and RP oligomers. A very similar pattern was obtained using the DP2 sequence oligomer giving an amplified fragment of 108 bp. β_2-microglobulin expression served as control. The oligodeoxynucleotides primers and probes used in the RT-PCR reaction are reported in Table 1.

Results

Treatment of Proliferating HL60 Cells with a Specific c-fes AS Oligomer and Differentiation Inducers. As shown in Fig. 1, after 96 h of treatment with the *c-fes* AS oligomer, the corresponding mRNA is not detectable by the RT-PCR technique. However cell growth does not appear to be modified, since the proportion of cells in G$_1$, S, and G$_2$ phases of the cell cycle is not altered by antisense treatment (Fig. 2) and the cell doubling time is 23 h, i.e., the same observed in untreated and *c-fes* S oligomer-treated HL60 cells (data not shown). The differentiation of HL60 cells is also not modified after the disappearance of *c-fes* mRNA when TPA and Vit. D3 are used as inducers, although the TPA-treated cells show a reduced adherence capability. On the contrary, a completely different result is obtained when the *c-fes* AS oligomer-treated

Table 1. *Nucleotide Sequences of Oligomers Used in the Antisense Strategy and in the RT-PCR Reaction*

| Gene	Deoxynucleotide sequence (5'-3')	Region	Mer	Amplified DNA fragment
				bp
c-fes S	GGCTTCTCTTCCGAGCTG	163–180	18	–
c-fes AS	CAGCTCGGAAGAGAAGCC	163–180	18	–
c-fes DP1	GGCTTCTCTTCCGAGCTG	163–180	18	258
c-fes DP2	TCCCTGCAGGACAGTGGG	313–330	18	108
c-fes RP	CAGCAAGCGGTTCAGGCC	557–574	18	
c-fes probe	TCCTGGGCTGAGATCACCAGCCAAACTGAG	525–554	30	
β_2-microglobulin DP	CTCGCGCTACTCTCTCTTTCT	1–21	21	162
β_2-microglobulin RP	TCCATTCTTCAGTAAGTCAACT	141–162	22	
β_2-microglobulin probe	CAGGTTTACTCACGTCATCCAGCAGAGAAT	55–84	30	
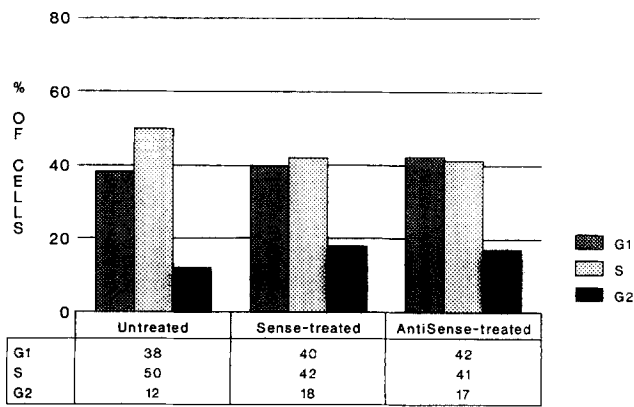
Figure 2. Effect of the c-fes S and AS oligomers on the distribution of HL60 cells among the different cell cycle phases after 120 h of incubation. Untreated cells are also reported. The doubling time of HL60 cells in the presence of S or AS c-fes oligomers was ~23 h, not unlike the doubling time of untreated HL60 cells.
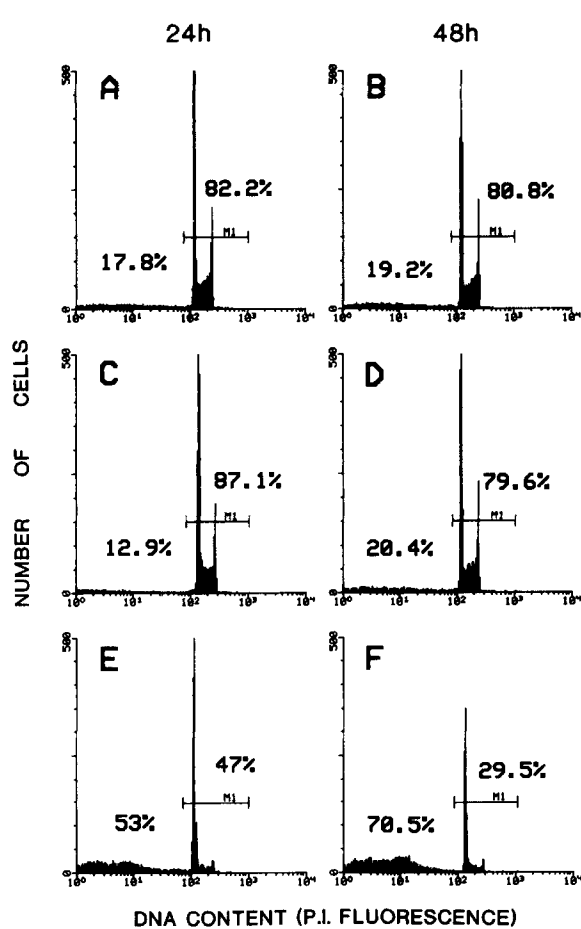
Table 2. *Effect of the c-fes AS Oligomer Treatment of the HL60 Cells on the 2C/4C Ratio after 24 and 48 h of Differentiation Induction*

Time	Sense	Antisense	Control
24 h	4,1	17,4	2,4
48 h	3,2	11,5	2,3

cells are induced to granulocytic differentiation by RA-DMSO. Albeit maintaining their immature characteristics, the cells begin to die after a few hours, and >70% of cell death is observed after 48 h (Fig. 3). No evidence of morphological and functional granulocytic differentiation was ever observed before apoptotic death. This appears to exclude the possibility that antisense treatment merely accelerates the differentiation process. It is worthwhile to point out that the evaluation of the 2c/4c ratio indicates that at 24 and 48 h of c-fes
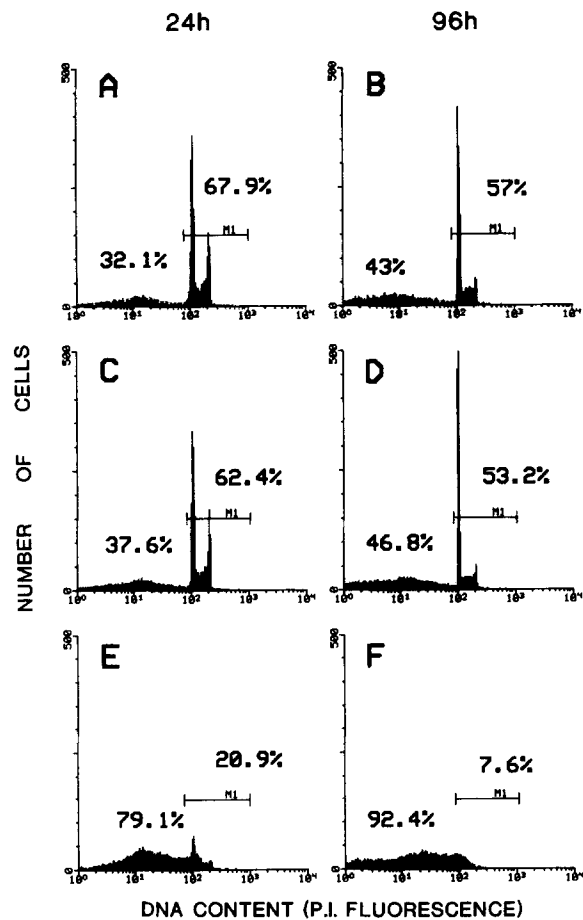

Figure 3. DNA fluorescence of PI-stained HL60 cells after 24 and 48 h of incubation under different experimental conditions. DNA histograms (log scale) of HL60 cells induced to differentiate with RA-DMSO (A and B), in presence of c-fes S oligomer (C and D) or c-fes AS oligomer (E and F), for 24 (left) and 48 h (right).

Figure 4. DNA fluorescence of PI-stained leukemic promyelocytes of APL after 24 and 96 h of incubation under different experimental conditions. DNA histograms (log scale) of APL cells induced to differentiate with RA-DMSO (A and B), in presence of c-fes S oligomer (C and D) or c-fes AS oligomer (E and F), for 24 (left) and 96 h (right).

AS oligomer treatment, there is a sharp increase in the proportion of cells in the G_1 phase of the cell cycle (Table 2).

Effects of c-fes AS Oligomer and Differentiation Inducers on Fresh Leukemic Promyelocytes of APL. On fresh leukemic promyelocytes of APL, the treatment with c-fes AS oligomer and differentiation inducers has an effect very similar to that observed in HL60 cells. As shown in Fig. 4, ~90% of the cells die after 4 d, whereas 40–60% of the untreated or S-treated cells are still alive.

Morphological and Molecular Characteristics of the HL60 Cell Death after Treatment with c-fes AS Oligomer and Induction to Granulocytic Differentiation. As shown in Fig. 5, the cell death is characterized by premature pycnosis followed by karyorrhexis without lysis of the cell. DNA double-strand cleavage produces fragments of ~185–200 bp, synchronously with the compaction of chromatin observed morphologically. This DNA ladder pattern is evident in the DNA extracted after 24 and 48 h of treatment with RA-DMSO and c-fes AS oligomer (Fig. 6). On the contrary, high molecular weight DNA is found in HL60 cells treated with the differentiation inducers alone or in association with c-fes S oligomer.

Protective Effects of Hemopoietic Growth Factors on Cell Death Induced by Inhibition of c-fes Gene Expression in RA-DMSO-treated HL60 Cells. As previously reported, IL-3, IL-6, SCF, GM-CSF, and G-CSF were tested to examine their capability to compete with the apoptotic process induced by the inhibition of c-fes gene expression during granulocytic differentiation. The cytofluorometric analysis, carried out by the method described above, shows that IL-3, IL-6, and SCF have no protective effect (Fig. 7). A quite different result is observed when GM-CSF and G-CSF are added to the cell cultures (Fig. 8). As summarized in Fig. 9, ~70% of the cells are protected both by GM-CSF and G-CSF, and the protection lasts for >3 d, since at 120 h of culture, >40% of the cells are still alive. Each experiment was repeated three times, and the variability was never >10%. On the contrary, treatment with IL-3, IL-6, and SCF does not result in any protective effect. Table 3 shows that IL-3, IL-6, and SCF are unable to avoid the increase of the 2c/4c ratio, although this increase is delayed. G-CSF and GM-CSF, on the contrary, leave this ratio unmodified and very similar to controls. These results confirm that an early consequence of the induction of apoptosis is the increase in the 2c/4c ratio.

Discussion

In this work we demonstrate that the loss of cell viability that occurs during the in vitro differentiation of myeloid cells, after the complete inhibition of the c-fes gene expression and treatment with RA-DMSO, is due to activation of pro-
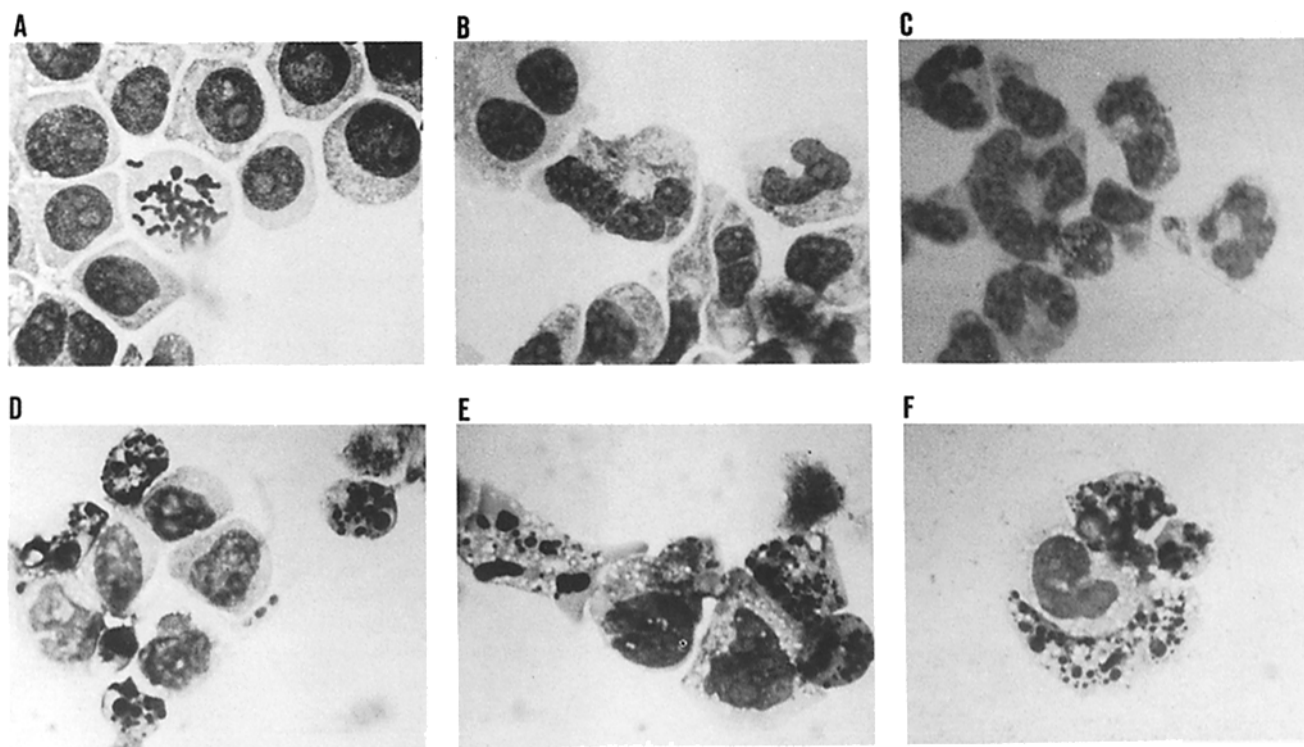

Figure 5. Morphological characteristics of HL60 cell death after treatment with c-fes AS oligomer and induction to granulocytic differentiation. (*A*) Proliferating HL60 cells after 5 d of treatment with c-fes antisense oligomer; (*B*) terminal differentiation with granulocytic morphology observed when the HL60 are induced to differentiate with RA-DMSO for 5 d; (*C*) terminal differentiation with granulocytic morphology observed when HL60 cells, pretreated for 5 d with c-fes S oligomer, are induced to differentiate with RA-DMSO for 5 d in presence of the same concentration of c-fes S oligomer; (*D–F*) progressive cell death of HL60 cells induced to differentiate with RA-DMSO in presence of c-fes AS oligomer for 24 (*D*), 48 (*E*) and 96 h (*F*). ×770.
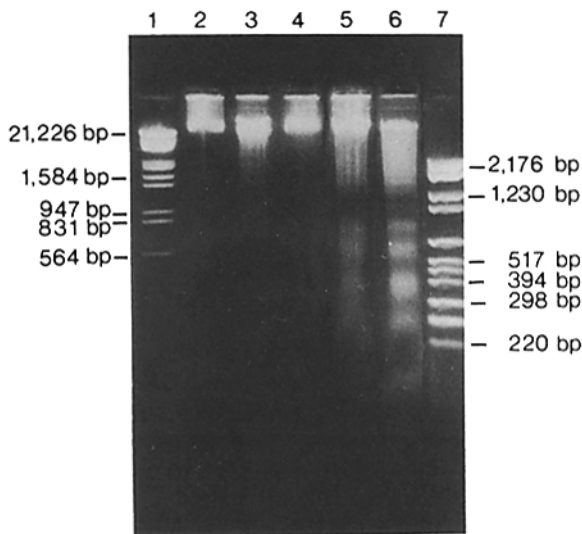
Figure 6. Agarose gel electrophoresis of DNA recovered from untreated and c-fes oligomer–treated HL60 cells. Lane 1, λ phage DNA digested with restriction endonucleases EcoRI and HindIII (DNA molecular weight marker III; Boehringer, Mannheim, Germany); lane 2, high molecular weight DNA extracted from proliferating HL60 cells; lanes 3 and 4, high molecular weight DNA extracted from HL60 cells induced to differentiate with RA-DMSO for 24 and 48 h, respectively, in the presence of c-fes S oligomer; lanes 5 and 6, fragmented DNA extracted from HL60 cells induced to differentiate with RA-DMSO for 24 and 48 h, respectively, in the presence of c-fes AS oligomer; lane 7, pBR328 digested with restriction endonucleases BglI and HinfI (DNA molecular weight marker VI; Boehringer).
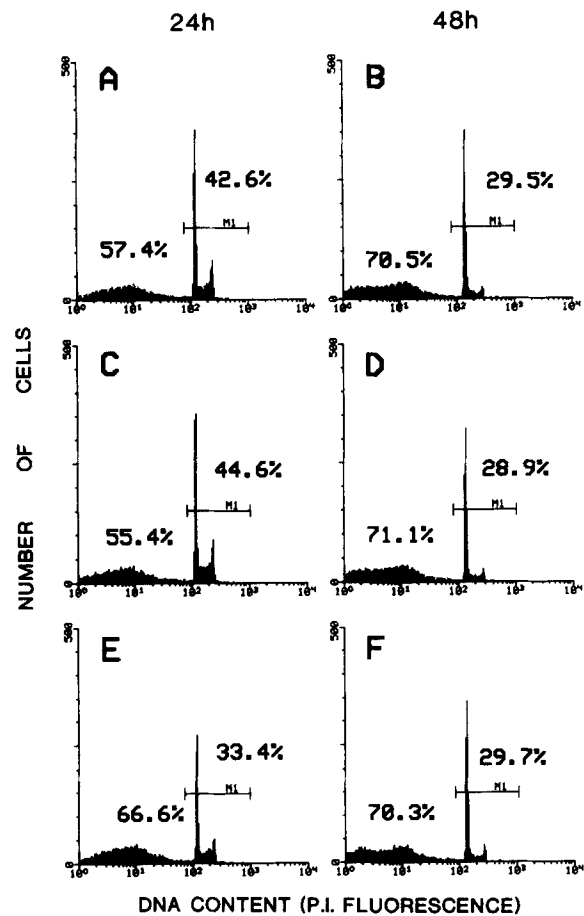

Figure 7. DNA fluorescence of PI-stained HL60 cells after 24 and 48 h of incubation under different experimental conditions. DNA histograms (log scale) of HL60 cells induced to differentiate with RA-DMSO in presence of c-fes AS oligomer and treated with IL-3 (A and B), IL-6 (C and D), and SCF (E and F) for 24 (left) and 48 h (right).

grammed cell death rather than to an accelerated cell differentiation. In fact, cytospins prepared at several different intervals from 24 to 96 h of c-fes AS oligomer and inducers treatment never show the presence of cells with differentiated granulocytic morphology. Cytochemical studies (data not shown) have not allowed us to obtain any evidence of progress toward granulocytic or monocytic differentiation. The morphological and molecular aspects of the effect of c-fes inhibition in HL60 differentiating cells suggest that the precocious cell death is the consequence of an apoptotic process. Strong support for this conclusion is brought by the observation that GM-CSF and G-CSF promote cell survival in differentiating HL60 cells by interfering with the inactivation of the c-fes proto-oncogene and thus suppressing apoptosis (43, 44). The molecular mechanism involved in the apoptotic process induced by c-fes inhibition needs to be identified. The apoptotic cell death is induced very early during the granulocytic differentiation of myeloid cells treated with the AS c-fes oligomer, occurring largely 24–48 h after the differentiation induction. HL60 cells undergo spontaneous apoptosis after terminal differentiation at least 6 d after RA-DMSO treatment (45). Normal neutrophils also undergo apoptosis during inflammation, and it seems possible to conclude that apoptosis represents the physiological way of death in these cells (46). In our experimental conditions, an important question is represented by the functional state of the cells involved in

the process. In fact the function of the c-fes product seems to occur mainly in granulocytic cells out of cycle, since the c-fes mRNA has been detected in increasing amounts in the terminal stages of granulocytic differentiation (12–14). Furthermore, no effect on cell proliferation has been observed by the AS treatment of proliferating HL60 cells. The GM-CSF and G-CSF have no inhibitory effect on the growth of HL60 cells and no enhancing effect on differentiation (47). Therefore the protective effect of G-CSF and GM-CSF is presumably related to events occurring in the short period when the cells, still in cycle, are drawn to terminal differentiation. The observation that at 24–48 h of AS c-fes treatment there is a sharp decrease in the proportion of 4c cells suggests that, as shown in other experimental systems (48), precocious death of S, G_2, and M cells is prevalent in these experimental conditions. This fact, and not a direct arrest in the G_1 phase of the cell cycle, is, therefore, in our opinion the origin of the accumulation of 2c cells. The function of the c-fes product may be considered essential to permit the survival of myeloid cells during differentiation. Leukemic myeloid blast cells can
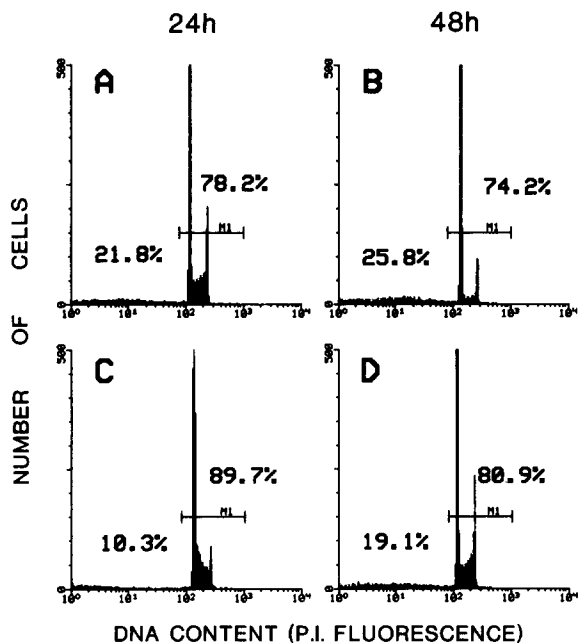
Figure 8. DNA fluorescence of PI-stained HL60 cells after 24 and 48 h of incubation under different experimental conditions. DNA histograms (log scale) of HL60 cells induced to differentiate with RA-DMSO in the presence of c-*fes* AS oligomer and treated with recombinant GM-CSF (*A* and *B*) and G-CSF (*C* and *D*) for 24 (*left*) and 48 h (*right*).

Figure 9. Concentration response of IL-3, IL-6, SCF, G-CSF, and GM-CSF on the viability of HL60 cells. The cells were preincubated for 5 d with c-*fes* AS oligomer and then induced to differentiate with RA-DMSO in the presence of the same oligomer and the various cytokines as described in Materials and Methods.

Table 3. *Effect of Different Growth Factors on the 2C/4C Ratio in HL60 Cells Treated with c-fes AS Oligomer and Differentiation Inducers*

Time	IL-3	IL-6	SCF	G-CSF	GM-CSF
24 h	3,2	3,6	4	4,9	3,5
48 h	12	13	10,8	4,2	5

survive for a long period of time in the peripheral blood with no significant proliferative activity and spontaneous differentiation (49). Our data suggest the possibility that this prolonged survival is in some way related to the sustained expression of the c-*fes* protooncogene in these cells (12–14). The c-*fes* protooncogene might represent another example of an oncogene whose function is to prevent premature cell death. The function might therefore be similar, in granulocytic cells, to that of the bcl-2 oncogene in pre-B cells (50, 51). Support for this hypothesis comes from the observation
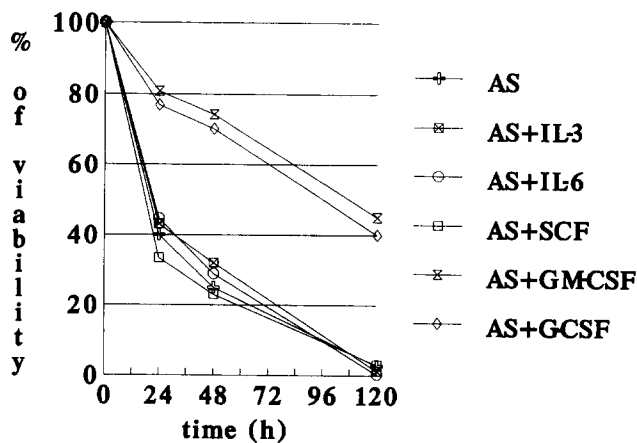
that the blast cells of promyelocytic leukemia are also sensitive to c-*fes* AS oligomers when induced to differentiate with RA. The possibility to induce programmed cell death of promyelocytic cells during the early stages of induced granulocytic differentiation (52) should therefore be explored.

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