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BRIEF COMMUNICATION

# Enhancement of cytosine arabinoside-induced apoptosis in human myeloblastic leukemia cells by NF- $\kappa$ B/Rel- specific decoy oligodeoxynucleotides

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The activity of NF- $\kappa$ B/Rel nuclear factors is known to inhibit apoptosis in various cell types. We investigated whether the subtraction of NF- $\kappa$ B/Rel activity influenced the response of 11 AML (M1, M2 and M4) patients' cells to AraC. To this end we used a phosphorothioate double-stranded decoy oligodeoxynucleotide (ODN) carrying the NF- $\kappa$ B/Rel- consensus sequence. Cell incubation with this ODN, but not its mutated (scrambled) form used as a control, resulted in abating the NF- $\kappa$ B/Rel nuclear levels in these cells, as verified by electrophoretic mobility shift assay (EMSA) of cells' nuclear extracts. We incubated the leukemic cells with AraC (32 or 1  $\mu$ M), in either the absence or presence of the decoy

or the scrambled ODN, and analyzed cell apoptosis. The spontaneous cell apoptosis detectable in the absence of AraC (<25%) was not modulated by the oligonucleotide presence in cell cultures. On the other hand, in 10 of the 11 samples tested, the decoy  $\kappa$ B, but not the scrambled ODN significantly ( $P < 0.01$  in a Student's *t* test) enhanced cell apoptotic response to AraC. Such an effect was particularly remarkable at low AraC doses (1  $\mu$ M). These findings indicate that NF- $\kappa$ B/Rel activity influences response to AraC in human primary myeloblastic cells, and suggests that the inhibition of NF- $\kappa$ B/Rel factors can improve the effect of chemotherapy in AML. Gene Therapy (2000) 7, 1234–1237.

**Keywords:** decoy oligodeoxynucleotides; NF- $\kappa$ B/Rel; human AML; apoptosis; chemotherapy

The NF- $\kappa$ B/Rel transcription factors<sup>1</sup> are dimers of proteins (p50/p105 or NF- $\kappa$ B1, p52/p100 or NF- $\kappa$ B2, p65 or RelA, c-Rel and RelB) containing an approximately 300 amino acid REL homology region. In cell cytoplasm, the NF- $\kappa$ B/Rel complexes are retained by inhibitors of the I $\kappa$ B ( $\alpha$ - $\epsilon$ ) family; cytokines, hormones and other stimuli can induce the phosphorylation and ubiquitin-mediated degradation of the I $\kappa$ B proteins, allowing the NF- $\kappa$ B/Rel dimers to reach the nucleus (reviewed in Ref. 1). In some cell types, including B cells, thymocytes and neurons, appreciable levels of NF- $\kappa$ B/Rel complexes can be detected also in nuclei.<sup>1,2</sup> Besides regulating a series of genes involved in inflammatory processes or cell adhesion,<sup>1,2</sup> NF- $\kappa$ B/Rel activities (specifically, p65- or c-Rel-containing dimers) can also inhibit apoptosis.<sup>3–13</sup> Indeed, in a number of lines of different origins, cell transfection with constructs expressing a 'superrepressor' I $\kappa$ B $\alpha$  (ie, mutated in the phosphorylation sites and hence resistant to degradation),<sup>3–5</sup> or the infection with a superrepressor I $\kappa$ B $\alpha$ -carrying adenovirus,<sup>9,11</sup> result in enhancing the apoptosis induced by TNF- $\alpha$ , ionizing radiations

or chemotherapeutic agents. On the other hand, the overexpression of p65 or c-Rel can protect the cells from apoptosis.<sup>3,10</sup> The inhibition of apoptosis appears to involve more than one NF- $\kappa$ B/Rel-regulated gene, including IAP caspase inhibitors,<sup>14,15</sup> the Bcl2- homolog Bfl-1/A1<sup>12,13</sup> and possibly others.<sup>8,16,17</sup>

The apoptosis-controlling activity of NF- $\kappa$ B/Rel factors supports anti-tumor strategies based on inhibition of NF- $\kappa$ B/Rel. Indeed, NF- $\kappa$ B/Rel inhibitors can be expected to increase tumor cell sensitivity to apoptosis, leading to enhanced effects of chemotherapeutic compounds.<sup>2,4,9,18–20</sup> For the block of transcription factor activities, the recently developed decoy strategy can represent a useful tool.<sup>21–25</sup> In several cell types, the introduction of *cis*-element double-stranded oligodeoxynucleotides (ODN) as decoy results in attenuating the authentic interaction between *trans*-factors and endogenous *cis*-elements, with subsequent modulation of gene expression.<sup>21–26</sup> By the effect of decoy ODN, the nuclear levels of NF- $\kappa$ B/Rel transcription factors can be downmodulated in primary cells<sup>26</sup> and the expression of NF- $\kappa$ B/Rel- inducible genes, such as cytokines or cell surface molecules, is inhibited.<sup>7,24,27–29</sup> To analyze whether the inhibition of NF- $\kappa$ B/Rel factors can influence leukemic cell response to pro-apoptotic chemotherapeutic agents, we investigated whether, in peripheral blood cells from acute myeloblastic leukemia (AML) patients, NF- $\kappa$ B/Rel- specific decoy ODN modulated cell

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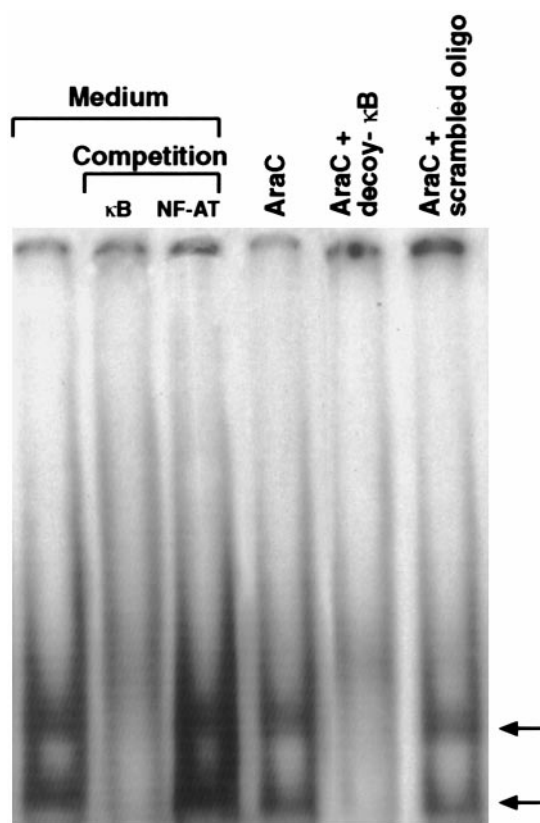
apoptosis induced by cytosine arabinoside (AraC). This study was aimed at verifying the survival-regulating property of NF- $\kappa$ B/Rel factors in human primary myeloblastic leukemia cells, and the effect of NF- $\kappa$ B/Rel decoy ODN combined with a specific chemotherapeutic agent.

We analyzed the levels of NF- $\kappa$ B/Rel nuclear complexes in peripheral blood leukemic cells obtained from AML (M1, M2 and M4) patients. At variance to normal human peripheral blood cells<sup>30</sup> and CD34<sup>+</sup> progenitors,<sup>26</sup> in which NF- $\kappa$ B/Rel complexes are poorly detectable in cell nuclei, we found the presence of NF- $\kappa$ B/Rel proteins in cell nuclei in nine of nine AML samples tested. In cells cultured with AraC, the NF- $\kappa$ B/Rel levels were not affected in five of the nine samples, and were only slightly reduced in the remaining four samples (results not shown). Representative results are shown in Figure 1. In AML cells incubated with either medium alone or AraC,

appreciable levels of DNA-protein complexes formed by nuclear proteins with a <sup>32</sup>P-labeled  $\kappa$ B oligo were evident in EMSA. Specifically, two bands (indicated by the arrows) were detected, in analogy with patterns observed in human peripheral blood T lymphocytes,<sup>30</sup> and probably corresponding to p50/p65 (upper arrow) and p50/p50 (lower arrow) dimers.<sup>1,30</sup> These bands were abolished when the nuclear proteins were incubated with the <sup>32</sup>P-labeled  $\kappa$ B oligo in the presence of a 50 $\times$  excess of a competing unlabeled  $\kappa$ B oligo, and not of an unrelated (NF-AT) oligo. Therefore, they specifically corresponded with the presence of NF- $\kappa$ B/Rel factors. When AML cells were incubated with AraC in the presence of an NF- $\kappa$ B/Rel- specific decoy phosphorothioate ODN,<sup>7</sup> and then the nuclear extracts were obtained, the NF- $\kappa$ B/Rel complexes were no longer detectable. Instead they persisted in the extracts of AML cells incubated with AraC in the presence of the scrambled oligo, used as control (Figure 1). Similar results were obtained by analyzing three different AML cell preparations. We concluded that cell incubation with the  $\kappa$ B decoy oligo specifically resulted in abating the NF- $\kappa$ B/Rel nuclear levels.

To investigate whether the subtraction of NF- $\kappa$ B/Rel nuclear complexes affected cell response to AraC, we incubated leukemic cells from 11 AML patients with the chemotherapeutic agent, in either the absence or presence of the decoy  $\kappa$ B or the scrambled oligonucleotide, and then analyzed cell apoptosis. The results are shown in Figure 2. Cultures without AraC displayed <25% of apoptotic elements; the apoptosis percentages were not significantly different in cultures with the decoy  $\kappa$ B or the scrambled oligonucleotide alone. On the other hand, in 10 (patients 1, 2, 3, 4, 5, 6, 7, 9, 10, 11) of the 11 samples tested, the presence of the decoy  $\kappa$ B oligonucleotide enhanced cell apoptotic response to AraC. In cultures with 32  $\mu$ M AraC (corresponding to a therapeutic dose of about 385 mg per application), cell apoptosis in the 11 samples was significantly enhanced ( $P=0.019$ ) by the decoy, but not the scrambled oligonucleotide. When AraC was used at a lower dose (1  $\mu$ M), the apoptotic response was even more strikingly raised ( $P=0.007$ ) by the decoy oligo. Four of the 11 samples tested with AraC 32  $\mu$ M (samples 5, 8, 9, 11) did not show any significant increase of their apoptotic response in the presence of the  $\kappa$ B decoy oligo. However, three of those four samples (samples 5, 9, 11) did show a significant effect of the decoy oligo at the lower AraC dose (1  $\mu$ M) (Figure 2). In five (patients 1, 5, 9, 10, 11) of the seven samples tested with both doses of AraC, apoptosis was raised to a level higher than that observed with 32  $\mu$ M AraC alone.

Therefore, NF- $\kappa$ B/Rel- specific decoy, but not scrambled ODN abated the nuclear levels of NF- $\kappa$ B/Rel factors and enhanced AraC induced apoptosis of AML cells. The lack of induction of AML cell death by NF- $\kappa$ B/Rel inhibition, in the absence of apoptosis-triggering agents, is consistent with findings concerning other cell types, either normal<sup>26</sup> or neoplastic.<sup>7,9,25</sup> These results concur in indicating that NF- $\kappa$ B/Rel activity is not required for maintaining basal cell survival. Instead, NF- $\kappa$ B/Rel factors can induce the expression of gene(s) whose products inhibit apoptotic events (caspase inhibitors, Bcl-2 homologs, etc).<sup>8,12-17</sup> In this respect, the constitutive nuclear NF- $\kappa$ B/Rel activities in some neoplastic cells,<sup>31,32</sup> including AML blasts, could represent one of the factors contributing to the transformed state, by

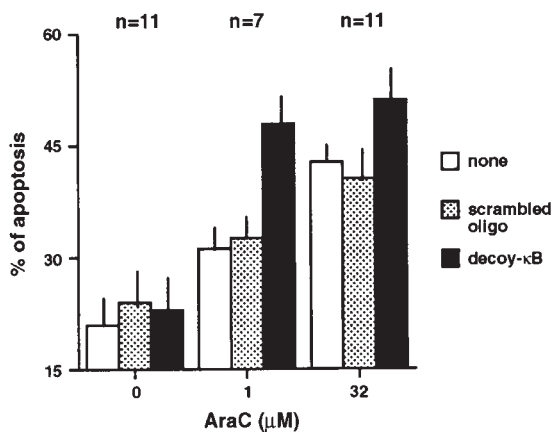


**Figure 1** Peripheral blood heparinized samples (>80% blasts) were obtained from AML (M1, M2 and M4) patients before the start of therapy and isolated by centrifugation through Ficoll-Hypaque (ICN Flow, Opera, Italy) density gradient at 400 g for 30 min. Cells (10<sup>6</sup>/ml) were incubated in RPMI 1640 medium supplemented with 10% FCS, in the absence or presence of cytosine arabinoside (AraC; Pharmacia & Upjohn, Puurs, Belgium) (32  $\mu$ M), and phosphorothioate oligodeoxynucleotides, corresponding to the  $\kappa$ B consensus sequence (5'-CCTTGAA GGGATTTCCCTCC-3')<sup>7</sup> or its mutated (scrambled) form (5'-CCTGTACCATTGTTAGCC-3') (Primm Srl, Milan, Italy) (5  $\mu$ M). After 20 h, nuclear extracts were obtained.<sup>30</sup> Nuclear proteins (3  $\mu$ g) were incubated with a <sup>32</sup>P-labeled  $\kappa$ B oligonucleotide (2  $\times$  10<sup>4</sup> c.p.m.) at room temperature for 20 min, in the presence of 1  $\mu$ g poly(dI-dC), in 20  $\mu$ l of a buffer consisting of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT and 5% glycerol. Where indicated, a 50 $\times$  excess of a competing unlabeled oligo ( $\kappa$ B or NF-AT) was added in the incubation mixture. Protein-DNA complexes were separated from free probe on a 6% polyacrylamide gel, run in 0.25 $\times$  Tris borate buffer at 200 V for 3 h at room temperature.

Patient n.	% of apoptosis			
	AraC 1 $\mu$ M		AraC 32 $\mu$ M	
	$\kappa$ B decoy		$\kappa$ B decoy	
	-	+	-	+
1	38.6 $\pm$ 0.7 <sup>a</sup>	63.7 $\pm$ 1.4	41.2 $\pm$ 1.4	53.7 $\pm$ 1.3
2	N.T. <sup>b</sup>	N.T.	27.0 $\pm$ 0.1	46.2 $\pm$ 3.9
3	N.T.	N.T.	59.0 $\pm$ 0.5	73.2 $\pm$ 8.6
4	N.T.	N.T.	40.7 $\pm$ 0.1	52.7 $\pm$ 5.1
5	30.6 $\pm$ 2.7	55.5 $\pm$ 6.1	49.7 $\pm$ 4.4	49.0 $\pm$ 5.4
6	39.6 $\pm$ 0.2	51.6 $\pm$ 0.4	59.0 $\pm$ 0.1	64.2 $\pm$ 1.9
7	N.T.	N.T.	34.7 $\pm$ 1.4	62.5 $\pm$ 2.4
8	17.5 $\pm$ 2.5	17.5 $\pm$ 2.8	39.6 $\pm$ 0.1	37.2 $\pm$ 2.7
9	25.2 $\pm$ 0.1	57.8 $\pm$ 4.0	41.1 $\pm$ 0.2	40.1 $\pm$ 0.1
10	36.0 $\pm$ 1.3	49.3 $\pm$ 3.3	40.0 $\pm$ 2.0	45.7 $\pm$ 5.3
11	30.4 $\pm$ 0.4	40.3 $\pm$ 0.9	39.7 $\pm$ 0.1	38.0 $\pm$ 2.8

<sup>a</sup> mean  $\pm$  S.D.

<sup>b</sup> not tested



**Figure 2** AML cells ( $10^6/ml$ ) were incubated in RPMI 1640 medium supplemented with 10% FCS, in the absence or presence of AraC and the  $\kappa$ B decoy or scrambled oligonucleotide (5  $\mu$ M). After 3 days, cell apoptosis was analyzed by propidium iodide incorporation and flow cytometry as described.<sup>35</sup> The *P* values were calculated using a Student's paired *t* test, GB-Stat 5.0 for Macintosh (Dynamic Microsystems, Silver Spring, MD, USA).

contrasting apoptogenic stimuli – such as those delivered by the immune system. Furthermore, NF- $\kappa$ B/Rel factors can be responsible for reducing cell responsiveness to cytotoxic agents. In fact, the inhibition of NF- $\kappa$ B/Rel enhanced AML cell response to AraC, and particularly amplified the effect of suboptimal doses of the drug. These findings support the possibility, suggested for different types of tumors,<sup>9</sup> that inhibitors of NF- $\kappa$ B/Rel activity can enhance leukemic cell sensitivity to chemotherapy.

The decoy ODN can represent a useful tool for manipulating the levels of NF- $\kappa$ B/Rel in primary cells. They are more specific than proteasome inhibitors or nuclear localization signals (NLS)-carrying peptides, have a biological effect *in vivo*<sup>24,25,29</sup> and their active concentration is comparable with that used for the systemic injection of other phosphorothioate oligonucleotides in phase I trials.<sup>33,34</sup> Furthermore, since the NF- $\kappa$ B/Rel activity is not apparently indispensable for the survival of normal cells, including human primary CD34<sup>+</sup> hematopoietic progenitors,<sup>26</sup> NF- $\kappa$ B/Rel- specific inhibitors, such as ODN, could be particularly useful for improving bone

marrow purging strategies *ex vivo* and supporting *in vivo* antineoplastic programs.

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