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Report

Increased sensitivity to cytosine arabinoside in human leukemia by c-*raf*-1 antisense oligonucleotides

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c-*raf*-1, a cytoplasmic serine/threonine protein kinase, plays an important role in mitogen- and damage-responsive cellular signal transduction pathways. Expression of c-*raf*-1 modifies cell growth, proliferation and survival. Although expression of c-*raf*-1 has been studied in several tumors, the role of c-*raf*-1 in leukemia is so far unclear. We examined the expression of c-*raf*-1 in the human leukemia cell lines U937 and K562, and in a cytosine arabinoside (Ara-C)-resistant cell line (K562AC) derived from K562. Expression of c-*raf*-1 was increased in U937 and in Ara-C-resistant K562AC cells compared with the parental cells. We then investigated whether inhibition of c-*raf*-1 expression by antisense oligonucleotides increases the sensitivity to Ara-C in U937 and K562AC cells. Antisense oligonucleotides for c-*raf*-1 inhibited expression of c-*raf*-1 mRNA, but did not affect cell growth and increased sensitivity to Ara-C but not to other drugs such as adriamycin, VP-16 or vincristine. These results suggest that c-*raf*-1 is one of the factors involved in Ara-C resistance in leukemia and lend weight to the case for development of anti-cancer therapeutics involving oncogene-targeted antisense oligonucleotides. [© 2001 Lippincott Williams & Wilkins.]

Key words: Antisense oligonucleotides, c-*raf*-1, cytosine arabinside, leukemia.

Introduction

c-*raf* genes encode for a family of cytoplasmic proteins (A-*raf*, B-*raf* and c-*raf*-1) with associated serine/threonine kinase activities.[1] The proto-oncogene c-*raf*-1 is an important mediator of signal transduction pathways involving cell growth, transformation and differentiation.[2] c-*raf*-1 is expressed in many human tissues, suggesting that c-*raf*-1 may be activated as an oncogene in carcinogenesis.[3] However, it is not yet known whether c-*raf*-1 is expressed in leukemia, since a variant of this gene was found in lymphoma patients.[4] Further, in terms of the relationship between c-*raf* and drug resistance, inhibition of RNA synthesis prevents Raf-1 activation and *bcl*-2 phophorylation, suggesting that an intermediate protein acts upstream of Raf-1 in the microtubule damage-activating pathway.[5] It has also been reported that expression of the c-*raf*-1 gene significantly enhanced the activity of the MDR1 promoter.[6,7] Another report indicated that although treatment of human U937 myeloid leukemia cells with phorbol ester (TPA) is associated with activation of the Raf-1 kinase, there was no detectable decrease in cells resistant to TPA.[8] However, to our knowledge, it has not been demonstrated that c-*raf*-1 is associated with drug sensitivity.

Antisense oligonucleotides targeted against c-*raf*-1 kinase resulted in potent anti-proliferative and anti-tumor effects.[9] Downregulation of c-*raf*-1 expression by antisense oligonucleotides inhibited BCR/ABL-dependent growth of chronic myelogenous leukemia cells and growth factor-dependent proliferation of normal hematopoietic progenitors, as did inhibition of c-*raf*-1 activity by its dominant-negative mutants.[10] Furthermore, sensitizing effects of these antisense oligonucleotides have also been reported in radioresistant tumors.[11,12] Thus, in this study to define the role of *raf*-1 in the mechanisms of drug sensitivity, we first investigated expression of c-*raf*-1 in leukemia cell lines and drug-resistant cell lines. Then, we investigated whether antisense oligonucleotides targeted against c-*raf*-1 kinase could reverse drug resistance in leukemia.

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Materials and methods

Chemicals

Cytosine arabinoside (Ara-C) was purchased from Nippon Sinyaku (Tokyo, Japan). 3-[4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), adriamycin (ADR) and vincristine (VCR) were obtained from Sigma (St Louis, MO), and etoposide (VP-16) from Bristol-Myers Squibb (Tokyo, Japan).

Cells

K562 and U937 were obtained from the cell bank at Tohoku University, and K562AC was established as a clone resistant to Ara-C.[13] These cell lines were maintained in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco) under 5% CO_2 in air at 37°C. To determine the cytotoxicity of drugs, 50% inhibition of cell growth (IC_{50}) values for these cells was determined by a dye reduction assay using MTT.

Synthesis of antisense oligonucleotides

Antisense and control oligonucleotides against the c-*raf*-1 gene were synthesized on the basis of the c-*raf*-1 mRNA sequence, as described previously.[14] The sequences of the antisense (ISIS-5132) and mismatched control analog oligonucleotides against for c-*raf*-1 were 5′- TCC CGC CTG TGA CAT GCA TT-3′ (antisense, ISIS-5132) from position 243 and 5′-TCC CGC GCA CTT GAT GCA TT-3′ (mismatch). Phosphorothioate was then conjugated to the synthesized antisense and sense oligonucleotides at all sequences during automated synthesis (Perkin-Elmer, Foster City, CA), and oligonucleotides were purified by preparative reverse-phase HPLC.

Northern blot analysis

Total RNA was isolated from cells with the guanidium isothiocyanate method and 20 μg of total RNA was separated by electrophoresis on an 8% polyacrylamide/7 M urea denaturing gel. This was transferred with blotting onto a Hybound-N nylon membrane (Ammersham Life sciences, Arlington Heights, IL). The blots were hybridized with cDNA probes for c-*raf*-1[14] and β-actin that had been labeled with [^{32}P]dCTP by nick translation, and then washed under high stringency conditions. The bands were detected by quantification of radioactivity with a Bio-Image Analyzer (BAS2000; Fiji Film, Tokyo, Japan).

Growth studies

For the colony assay, cells were plated at 2.5×10^3 cells/well in 96-well microtiter plates and grown in 0.3% agar with RPMI1640 medium containing 1 or 20% FBS. Antisense or mismatched oligonucleotides (final concentration 2 μM) were added into the medium 1 h after plating of cells. After 48 h of treatment with antisense or mismatched oligonucleotides, the colonies were detected with Giemsa dye stain. To determine the rate of [^{3}H]thymidine incorporation into acid-insoluble material, cells were grown for 48 h, incubated for 2 h with [^{3}H]thymidine (0.15 μCi/mmol), and then washed, acid precipitated and counted as previously described.[15]

Results

We had previously determined the expression of the c-*raf*-1 gene in several human leukemia cell lines; however, almost all cell lines showed lower levels of expression compared with the U937 cell line. First, treatment of U937 cells with antisense oligonucleotides (ISIS-5132) caused decreased expression of c-*raf*-1 at 48 h, but no change was seen with mismatched control oligonucleotides (mismatches) (Figure 1). Treatment with the antisense oligonucleotides was compared in a kinetic analysis of the mRNA to determine the relative potency and duration of effect. A time-dependent reduction of c-*raf*-1 expression was observed following transfection of cells with ISIS-5132 (data not shown). The dose dependency and sequence specificity of the oligonucleotide-mediated inhibition of c-*raf*-1 expression were assessed using control oligonucleotides with mismatches. Dose-dependent decreases of c-*raf*-1 expression were observed with antisense oligonucleotides but not mismatched control oligonucleotides (data not shown). Secondly, similar treatment of K52AC cells with antisense oligonucleotides (ISIS-5132) also caused decreased expression of c-*raf*-1, which again was not changed by mismatched control oligonucleotides (mismatches) at 48 h (Figure 2). Further, the cell numbers with antisense oligonucleotides treatment and no treatment control were not changed at 1, 2, 12, 24 and 48 h, suggesting that it had no cytotoxic effects (data not shown).

Cell counting is a reliable direct indicator of proliferative activity. Cell numbers were not affected in either cell line after 24 and 48 h treatment with oligonucleotides (data not shown). The colony assay and the rate of [^{3}H]thymidine incorporation as an indirect indicator of cell proliferation were performed in both cell lines after 48 h treatment with oligonu-

Figure 1. Northern blot analysis of gene expression in the U937 cell line after treatment with antisense or control oligonucleotides for 48 h. Upper column: c-*raf*-1 gene; lower column: β-actin. The lanes are as follows: lane 1, U937; lane 2, U937 with antisense oligonucleotides (10 μM); lane 3, U937 with control oligonucleotides (10 μM).

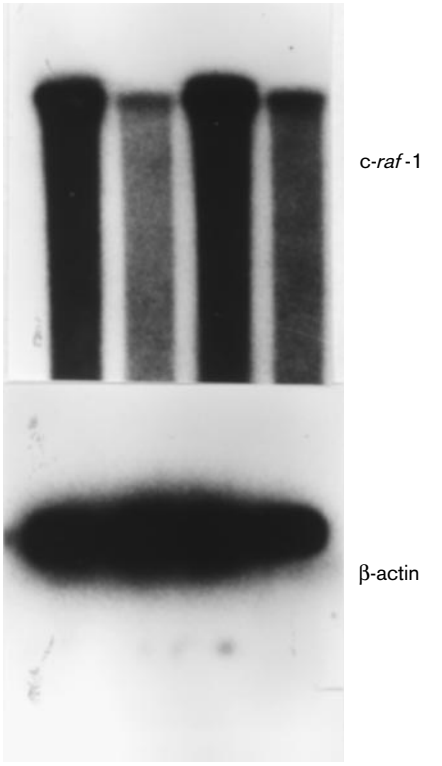

Figure 2. Northern blot analysis of gene expression in the K562 and K562AC cell lines after treatment with antisense or control oligonucleotides for 48 h. Upper column: c-*raf*-1 gene; lower column: β-actin. The lanes are as follows: lane 1, K562; lane 2, K562AC; lane 3, K562AC with antisense oligonucleotides (10 μM); lane 4, K562AC with control oligonucleotides (10 μM).

cleotides. However, the colony numbers and the incorporation rate of [^{3}H]thymidine were not changed (Table 1).

We then examined the effect of antisense oligonucleotides against c-*raf*-1 on sensitivity to Ara-C. The results of four independent tests showed a 2- to 3-fold increase in sensitivity to Ara-C after treatment with antisense oligonucleotides, but not after treatment with mismatched control oligonuclotides (Table 2). However, the sensitivity to other drugs such as ADR, VCR or VP-16 was not changed in any cell lines after treatment.

Table 1. Growth characteristics of cell lines

	No. of colonies (serum: 1%/20%)	[^{3}H]thymidine incorporation (%)
U937	28±9 /136±43	100
U937 with antisense	24±8 /141±29	96±6
U937 with control	18±10/154±27	94±5
K562	34±11/205±38	100
K562AC	42±16/220±23	102±8
K562AC with antisense	32±19/188±35	94±7
K562AC with control	34±10/213±24	96±8

For [^{3}H]thymidine incorporation, the results are expressed as a percentage relative to the untreated control U937 cell or K562 cell as 100%. Values are given as mean±SEM (n=5). There was no statistically significant difference in the results of the growth experiments

Discussion

In this study, we have examined the role of c-*raf*-1 expression on growth and Ara-C-mediated cytotoxicity in human leukemia cells. We found that antisense oligonucleotides against c-*raf*-1 markedly inhibited expression of c-*raf*-1 mRNA in leukemia cells, but did not affect cell growth. The inhibitory effects of antisense oligonucleotides for c-*raf*-1 on overexpression of c-*raf*-1 in various diseases were mainly obtained

Table 2. Cell cytotoxicity (IC_{50}) to various drugs in cell lines

	Ara-C (μM)	ADR (nM)	VCR (nM)	VP-16 (μM)
U937	2.36±1.68	0.67±0.21	1.56±0.16	5.8±2.1
U937 with antisense	1.54±0.85	0.57±0.33	1.41±0.25	5.2±1.3
U937 with control	2.44±0.60	0.53±0.26	1.55±0.32	6.1±0.9
K562	1.96±0.48	0.34±0.27	0.88±0.55	4.8±2.2
K562AC	42.63±3.14	0.57±0.41	1.25±0.34	5.1±1.5
K562AC with antisense	16.42±8.32	0.51±1.02	1.17±0.82	6.4±3.0
K562AC with control	40.31±5.73	0.91±0.84	1.38±0.71	5.6±2.9

These values are given as mean±SEM ($n=5$). Statistical significance at the $p<0.05$ level is indicated by an asterisk.

on cellular signal transduction pathways.[9,16–20] However, *raf*-1 also has survival functions, indicating a possible role for *raf* antisense in the management of radioresistant malignancies, as demonstrated by its radiotherapeutic efficacy.[4] However, although c-*raf*-1 plays important roles in cell growth and proliferation, its role in drug sensitivity is so far unclear. In the present study we showed that antisense oligonucleotides against c-*raf*-1 had no effects on cell growth as measured by colony formation and DNA synthesis.

The focus in this study was whether the expression of c-*raf*-1 is related to drug sensitivity, particularly to Ara-C in leukemia. We demonstrated by using antisense oligonucleotides technology that c-*raf*-1 expression is associated with drug sensitivity to Ara-C. A study of sensitivity to Ara-C in leukemia observed that clinical resistance to Ara-C is associated with reduced expression of deoxycytidine kinase (dCK) mRNA in leukemia cells.[21] Nevertheless, data on the molecular events leading to Ara-C resistance are still sparse. Although expression of c-*raf*-1 enhanced the activity of the MDR1 promoter,[6] in the present study modification of c-*raf*-1 expression did not affect sensitivity to drugs such as ADR or VCR that are related to the MDR phenotype. To our knowledge, there are no previous reports directly demonstrating that c-*raf*-1 is related to drug sensitivity for Ara-C or that link inhibition of c-*raf*-1 expression by antisense oligonucleotides and drug sensitivity to Ara-C in human leukemia. We conclude that the inhibitory effect on c-*raf*-1 should be useful by causing increased sensitivity to Ara-C in leukemia. Therefore, the c-*raf*-1 oncogene expressed in leukemia may be an attractive target for tumor-specific therapy. Further, clinical trials of antisense oligonucleotides (ISIS-5132) for c-*raf*-1 have recently been reported in refractory malignancies for phase I,[22] in advanced cancer[23] and in ovarian cancer with mutated p53.[24] The preferential of targeting drug resistance may develop for antisense therapeutics.

Conclusion

The c-*raf*-1 oncogene plays an important role in cellular signal transduction pathways that mediate cell growth and proliferation. To evaluate the effect on drug sensitivity of a well-characterized *raf* antisense oligonucleotide, we examined whether expression of c-*raf*-1 is related to resistance to Ara-C in human leukemia. Modulation of gene expression by antisense oligonucleotides altered drug sensitivity to Ara-C in leukemic cells.

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