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Antitumor Activity of Synthetic Oligonucleotides with Sequences from cDNA Encoding Proteins of *Mycobacterium bovis* BCG

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Thirteen kinds of 45-mer or 30-mer synthetic oligonucleotides with sequences randomly selected from the cDNA encoding three kinds of protein of *Mycobacterium bovis* BCG were tested for their antitumor activity in a murine tumor system. Six out of the 13 single-stranded oligonucleotides which contained one or more hexameric palindromic sequences showed strong antitumor activity while the others without palindromic structure did not. Namely, repeated intralesional injections of 100 μg of the 6 oligonucleotides caused regression of the established tumor but the other 7 were ineffective. When tumor cells were mixed with 100 μg of an effective oligonucleotide and injected into mice, tumor growth was markedly suppressed. These results suggested that palindromic structure is essential for the antitumor activity of the synthetic oligonucleotides.

Key words: Antitumor activity — Mycobacterial gene — Synthetic DNA — Oligonucleotide

We have reported previously that a DNA-rich fraction extracted and purified from *Mycobacterium bovis* BCG, named MY-1, showed antitumor activity against various murine and guinea-pig tumors.[1,2] Recently, it was also found that MY-1 augmented natural killer (NK) cell activity and produced interferon(IFN)-α/β and -γ either *in vitro* or *in vivo*.[3,4] All of the biological activities were ascribed to DNA contained in MY-1.[1–4] Since MY-1 was proved to be nontoxic to experimental animals and humans, immunotherapeutic trials of MY-1 are under way.[5,6]

To clarify the mechanism responsible for such activities of MY-1, DNA, many kinds of 45-mer oligonucleotides, the sequences of which were randomly chosen from the known cDNA sequence of 3 different mycobacterial proteins, i.e., 64 kDa heat shock protein (Antigen A),[7] MPB-70[8] and α-Antigen,[9] were synthesized and their biological activities were tested *in vitro*.[10] The reason why such a molecular size of oligonucleotide was synthesized is that a major component of MY-1 is 45-mer single-stranded DNA.[10] The results obtained showed that some of the oligonucleotides were capable of stimulating mouse spleen cells to produce IFN-α/β and -γ, and augmented NK cells, while the others were ineffective.[10] Based on these facts, we tested the antitumor activities of these oligonucleotides, and compared them with their reported NK-augmenting activities.[10]

Female CDF1 mice, approximately 6 weeks old, were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka Pref.). IMC tumor, a carcinoma produced spontaneously in CDF1 mice and maintained as an ascitic tumor,[11] was provided by Dr. T. Ishizuka, Institute of Microbial Chemistry, Tokyo. Thirteen kinds of 45-mer and two 30-mer oligonucleotides, were synthesized as described previously.[10] Briefly, they were synthesized on an automated DNA synthesizer (Gene Assembler Plus: Pharmacia-LKB, Uppsala, Sweden), purified by ethanol precipitation followed by a gel-filtration chromatography, and then lyophilized. The names, the nucleotide residues in the cDNA, and the sequences of the oligonucleotides employed are shown in Table I.

To evaluate the antitumor activity of each oligonucleotide, 0.05 ml of a cell suspension containing 5×10^5 IMC tumor cells was mixed with 0.05 ml of test sample containing 100 μg of oligonucleotide, and inoculated intradermally into CDF1 mice. As a control, an equal volume of saline alone was used instead of the test sample. At 35 days after the inoculation, all mice were killed and the tumors were resected for weighing. As shown in Table II, when IMC tumor cells were pre-mixed with BCG-A4 or -A4a, the tumor growth was markedly suppressed, but BCG-A2 and -A4b did not significantly affect the tumor growth. Next, the effects of repeated injections of the oligonucleotides into established tumors were examined. IMC tumor cells (5×10^5) were inoculated intradermally into CDF1 mice. Test samples (100 μg each) dissolved in 0.1 ml of saline were injected into the tumor lesion twice a week from the 5th day of the tumor inoculation (Exp. I), or every other day from the 4th day after tumor inoculation (Exp. II) up to 6

Table I. The Sequences of Oligonucleotides Used

Name	Protein	Nucleotide residues	Base sequence
BCG-A1		327–371	AACGAGGGGC ATGACCCGGT GCGGGGCTTC TTGCACTCGG CATAG
BCG-A2		694–738	AAAAGAAGTG GGGTGCCCCC ACGATCACCA ACGATGGTGT GTCCA
BCG-A3		735–779	TCCATCGCCA AGGAGATCGA GCTGGAGGAT CCGTACGAGA AGATC
BCG-A4	65 kDa	813–857	ACCGATGACG TCGCCGGTGA CGGCACCACG ACGGCCACCG TGCTG
BCG-A4a	(Antigen-A)	813–842	ACCGATGACG TCGCCGGTGA CGGCACCACG
BCG-A4b		828–857	GGTGA CGGCACCACG ACGGCCACCG TGCTG
BCG-A5		1145–1189	TATGCGGTTC GACAAGGGCT ACATCTCGGG GTACTTCGTG ACCGA
BCG-A6		1552–1596	ACGAGACCAC CATCGTCGAG GGCGCCGGTG ACACCGACGC CATCG
BCG-A7		1962–2006	GCCGAGAAGG TGCGCAACCT GCCGGCTGGC CACGGACTGA ACGCT
BCG-A8		2371–2415	ACCGAGAACA GCCACGCAGT CGTGTAGGCA ACCTTTGGCC GCTGT
BCG-M1	MPB70	1–45	GGCGATCTGG TGGGCCCGGG CTGCGCGGAA TACGCGGCAG CCAAT
BCG-M3		410–455	ACGCCGACGT CGTCTGTGGT GGGGTGTCTA CCGCCAACGC GACGG
BCG-α1	α-Antigen	348–392	CGACTACAAC GGCTGGGATA TCAACACCCC GGCGTTCGAG TGGTA

The underlines show the palindromic sequences. 64kDa: 64kDa heat shock protein (Antigen-A), 2431 bp.[7] MPB70: MPB70 protein, 492 bp.[8] α-Antigen, 1165 bp.[9]

Table II. Suppression of IMC Tumor Growth in CDF1 Mice by the Oligonucleotides

Exp.	Sample	No. of mice without tumor[a]/ No. of mice tested	Tumor weight (mean±SD) (g)	Growth inhibition[b] (%)	Student's t test
I	Saline	0/3	1.09±0.64	0	
	BCG-A2	0/3	0.48±0.28	56.0	NS[c]
	BCG-A4	3/3	0	100.0	$P<0.05$
II	Saline	0/5	1.13±0.53	0	
	BCG-A2	0/5	0.82±0.20	27.0	NS
	BCG-A4	1/5	0.28±0.20	75.0	$P<0.05$
	BCG-A4a	1/5	0.36±0.26	68.0	$P<0.05$
	BCG-A4b	0/5	1.18±0.27	−4.0	NS

a) Number of mice in which tumor growth was completely suppressed.
b) Growth inhibition (%) = (1 − test/control) × 100.
c) NS, not significant.

times. At 35 days (Exp. I) or 27 days (Exp. II) after the tumor inoculation, all the mice were killed and the tumors were resected for weighing. The results are shown in Table III. Six of the 11 oligonucleotides tested, i.e., BCG-A3, -A4, -A6, -A7, -M3 and -α1, inhibited the tumor growth significantly, while the others did not. As shown in Fig. 1, the antitumor activity of the oligonucleotides correlated well with the NK-augmenting activity. Although the data are not shown, these activities also correlated with the IFN-inducing activities.

It is noteworthy that the inactive oligonucleotides, BCG-A1, -A2, -A5 and -A8, do not contain a hexameric palindromic sequence, while the active oligonucleotides do possess such a sequence, i.e., GACGTC, GGCGCC or TGCGCA. In Table I, the sequences are underlined. The only exception is that an inactive oligonucleotide BCG-M1, contains an overlapping palindromic sequence (GGGCCCGGG).

When six bases of an inactive oligonucleotide, such as BCG-A4b, were substituted with a palindromic sequence of GACGTC, GGCGCC or TGCGCA, but not with GGGCCCGGG, the oligonucleotide acquired the ability to augment NK cells and to produce IFNs (manuscript submitted), so it seems clear that particular hexameric

Table III. Regression of IMC Tumor in Oligonucleotide-treated CDF1 Mice

Exp.	Sample	No. of cured mice[a]/No. of mice tested	Tumor weight (mean ± SD) (g)	Growth inhibition[b] (%)	Student's *t* test
I	Saline	0/5	0.30±0.09		
	BCG-A2	0/5	0.13±0.15	56.7	NS[c]
	BCG-A4	4/5	0.02±0.04	93.3	$P<0.01$
II	Saline	0/7	1.45±0.85		
	BCG-A1	0/7	1.27±0.60	12.4	NS
	BCG-A3	1/5	0.42±0.37	71.0	$P<0.05$
	BCG-A5	0/7	0.96±0.41	33.8	NS
	BCG-A6	0/7	0.62±0.41	57.2	$P<0.05$
	BCG-A7	1/7	0.58±0.44	60.0	$P<0.05$
	BCG-A8	0/7	0.91±0.44	37.2	NS
	BCG-M1	0/7	1.00±0.58	31.0	NS
	BCG-M3	2/6	0.65±0.69	55.2	$P<0.05$
	BCG-α1	3/7	0.11±0.16	92.4	$P<0.01$
	BCG-DNA[d]	2/7	0.28±0.16	80.7	$P<0.01$

a) Number of mice in which tumor eventually disappeared.
b) Growth inhibition (%) = (1 − test/control) × 100.
c) NS, not significant.
d) DNA extracted from BCG by Marmur's method.[12]

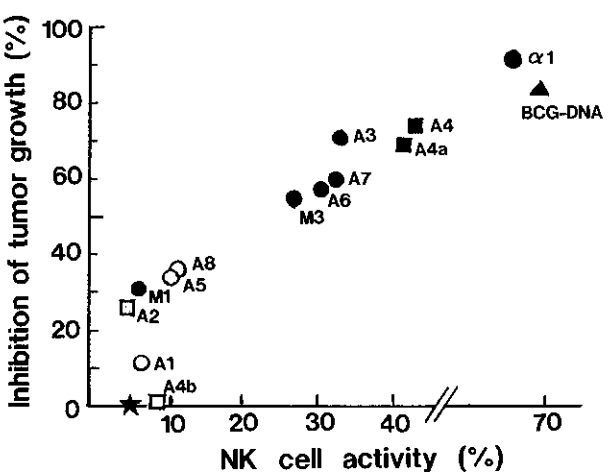

Fig. 1. Correlation between antitumor activity and NK cell-augmenting activity induced by the oligonucleotides. The values of tumor growth suppression (□ & ■) and regression (○ & ●) were obtained from Tables II and III, respectively. The values of NK cell activity induced by the oligonucleotides were taken from the previous paper.[10] In the experiments, normal mouse spleen cells were incubated with or without oligonucleotide (50 μg/ml) for 20 h at 37°C. The cytotoxicity towards YAC-1 cells was measured by 4-h ^{51}Cr-release assay at an E/T ratio of 50, and expressed by the following formula: %lysis = [experimental release (cpm) − spontaneous release (cpm)]/[total release (cpm) − spontaneous release (cpm)] × 100. Closed circles and squares (● & ■) represent the oligonucleotides with palindromic sequences and open circles and squares (○ & □) represent those without a palindromic sequence. For comparison, values obtained by cultivation with DNA fraction extracted from BCG by Marmur's method[12] (▲) and with medium alone (★) are presented.

palindromic structures are essential for expressing the biological activities of oligonucleotides, and also for antitumor activity. Further studies on the molecular mechanisms of the activities of oligonucleotides with particular base sequences are under way.

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