Environmental Mutagens & Carcinogens 22-2 : 65-69 (2002)

Biodistribution of [S-35] Labeled Antisense Oligodeoxynucleotides Increased Tumor Targeting With Microsphere Coinjection

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(Received March 12, 2002 / Accepted April 25, 2002)

ABSTRACT : **To elucidate the effect of microsphere coinjection on the administration of oligodeoxynucleotide (ODN), we have investigated biodistribution of [S-35]-labeled antisense ODN targeted to cAMP-dependent protein kinase (PKA) RI-α subunit in nude mice xenografted with WiDr (human colon cancer, ATCC CCL218). The strategy of using microsphere has been proposed for cancer treatment as a carrier of therapeutic ODN so that it could offer an advantage with respect to maintaining constant ODN levels in blood and obtaining higher therapeutic ODN concentration at tumor sites. Comparative biodistribution studies were performed in nude mice (female, 20 g of body weight, n = 4-6) xenografted with WiDr cancer cells, when 0.1 μCi (specific activity, 2.94 mCi/μmole) of [S-35]-labeled RI-α antisense ODN was injected alone or with microsphere (PLG-18, polylactic copolymer with cationic surfactant DDAB18). Peak tumor uptake of [S-35]-labeled ODN was significantly increased from 17.7% (at 6 h) of injected dose per gram of tissue (ID/g) to 42.5% (at 24 h) ID/g when microsphere was coinjected with ODN. The different biodistribution in the kidney accumulation (e.g., 100.2%ID/g for ODN alone and 54.9%/ID/g for microshpere coinjection) may contribute to higher blood concentration (e.g., 21.5%ID/ml for ODN alone and 37.5%ID/ml for microsphere coinjection) of radiolabeled ODN. Of importance is the fact that the whole body retention of radioactivity increased with microsphere coinjection from 50.8%ID/g to 68.0%ID/g after 24-h of injection. This decreased kidney accumulation and increased whole body retention of [S-35]-labeled ODN resulted in a significant improvement of ODN targeting to the tumor site. In conclusion, the coinjection of microsphere appears to be an important carrier system in vehiculation of antisense oligonucleotide to the tumor tissue *in vivo*.**

Keywords: microsphere, oligodeoxynucleotide, tumor, targeting, therapeutic

Introduction

Antisense oligodeoxynucleotides (AS-ODN) are short, single-strained, synthetic sequences complementary to the sequences of target mRNAs. These AS-ODN can be used to selectively inhibit the synthesis of a defined protein. Therefore, the use of AS-ODN may provide an important therapeutic approach for genetic, neoplastic, and infectious diseases, and block the synthesis of the protein responsible for pathologic disorders (Crooke, 1993; Greary *et al.*, 1997; Cho-Chung, 1999). However, therapeutic application of AS-ODN has been hampered by their rapid cleavage in the blood due to endonucleases and by their poor cellular uptake at tumor sites (Ferreiro, 2001).

The former problem has been improved by using phosphorothioate oligodeoxynucleotides (PS-ODN) and mixed-backbone oligodeoxynucleotides (MBO) (Agrawal, 1997; Rusckowski, 2000). The MBOs are the second-generation PS-ODN which contain a segment of 2'-O-methyl RNA or methylphosphonate ODN. The MBOs are more metabolically stable and are retained longer in tissues than PS-ODN: it means that it may be possible to administer them less frequently. The latter problem have been widely investigated by introducing gene delivery systems, such as viral vectors, cationic liposomes, nanoparticles, microemulsions, microencapsulated system, and complexes with polycations (Esposito,1999; Cortesi, 1999). The strategy of non-viral carriers of ODN, such as neutrals of cationic liposomes and polymeric microspheres, has been proposed to improve the cancer-cell targeting. Indeed, AS-ODN with liposomes and microparticles are believed to display better stability in the presence of nucleases and increased cell accumulation *in*

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vitro. *In vivo*, the microsphere could offer an advantage with respect to: 1) maintaining constant ODN levels in blood (Nastruzzi, 1993) and 2) obtaining higher therapeutic ODN concentration at tumor sites (Aynie, 1999; Carome, 1997).

The objectives of this study were to assess the biodistribution of [S-35]-labeled AS-ODN targeting cAMP-dependent protein kinase (PKA) RI-α subunit in nude mice xenografted with WiDr (human colon cancer, ATCC CCL218) and to compare the effect of microsphere coinjection mainly based on the time of administration and the size of tumor xenografted.

Materials and Methods

Synthesis of [S35]-labeled oligodeoxynucleotides

Antisense RI-α MBO is an 18-base RNA-DNA hybrid PS-ODN, 5'-[GCGU]GCCTCCTCAC[UGGC]-3'. This contains segments of four 2'-O-methylribonucleosides at both 5'- and 3'-ends (bracketed). Five contiguous phosphorothioate linkages from the 5'-end were labeled with [S-35] using previously published methods (Aynie, 1999). [S-35]-labeled MBO was administered to mice with the dose of 0.1 μCi (2.94 mCi/μmole).

Microspheres

Four types of microspheres, e.g., methacrylic copolymers with cationic surfactant DDAB18 (MSE-18), with cationic surfactant DDAB-12 (MSE-12), with cationic surfactant CTAB (MSE-CTAB), and polylactic acid copolymers with cationic surfactant DDAB18 (PLG-18), were synthesized: PLG-18 was selected for the biodistribution study after preliminary screening of tumor-growth inhibition. To obtain an appropriate complexation of DNA with microsphere, 0.2 mg of ODN sample was added to 1 mg of microsphere (5-times excess).

Cancer-cell xenograft

For studying the tumor-bearing mice model, athymic mice (female, 20 g of body weight, n = 4-6) were supplied by the National Cancer Institute (Frederick, MD, USA). To establish a tumor model for the biodistribution, WiDr (human colon cancer cells, ATCC CCL218) was cultured in Eagle's MEN with 10% fetal bovine serum up to 80% of confluence. The cells were removed from culture dishes by trypsinization, washed with phosphate buffered saline (PBS) three times, and then resuspended in PBS with the concentration of 15 million cells per ml. For xenograft, 0.1 ml of cell suspension, i.e., 1.5×10^6 cells per mouse, was injected subcutaneously into the right flank region. The mice were used 10-20 days after the injection, depending on the time point when weight of tumor reached 0.2, 0.4, 0.6 g, respectively. At the time of the study, the average mouse weight was 19.7±1.0 g.

Biodistribution

Comparative biodistribution studies of [S-35]-labeled MBO were performed in tumor-bearing mice. Mice at each time point were euthanized by CO_2 inhalation and sacrificed by cardiac puncture. Each organ or tissue, including blood, liver, kidney, muscle, and tumor was weighed in total and in portion for radioactivity measurement (Teder, 1995). For quantification of radioactivity in tissues, 20-50 mg of each tissue was incubated overnight at room temperature, precipitated with 0.5 ml of 10% acetic acid, mixed with 8 ml of scintillation cocktail solution, and measured for radioactivity using a α-scintillation counter. Measurement of whole body radioactivity was also performed by dose calibrator. On the basis of weight and radioactivity in each tissue, percentage of injected dose per gram (%ID/g) was calculated and normalized to the value of 20-g weighed mouse.

Statistical analysis

One-way Analysis of Variance (ANOVA) was performed to compare the statistical significance ($p<0.05$) between the control group and the experiment groups using a Sigma Stat (SPSS Science, IL).

Results

Microsphere coinjection

Comparing the effect of microsphere coinjection (Fig. 1), a peak tumor uptake of [S-35]-labeled MBO was significantly increased from 17.7 percent of injected dose per gram of tissue (%/ID/g) at 6 h to 42.5 %ID/g at 24 h. The different biodistributions in the kidney accumulation (e.g., 100.2 %ID/g for ODN alone and 54.9 %ID/g for microsphere coinjection) may reflect the blood concentration (e.g., 21.5 %ID/ml for ODN alone and 37.2 %ID/ml for microsphere coinjection) of radiolabeled ODN. Of importance is the fact that the whole body retention of radioactivity increased with microsphere coinjection from 50.8 %ID/g to 68 %ID/g after 24 h of injection. This lowered kidney accumulation and increased whole body retention of [S-35]-labeled ODN resulted in a significant improvement of ODN targeting at the tumor site.

Tumor-size variation

We tested the effect of microsphere coinjection on the variable size of tumors: 1) small-sized, 202±33 mg; 2) medium-sized, 387±32 mg; and 3) large-sized, 679±64

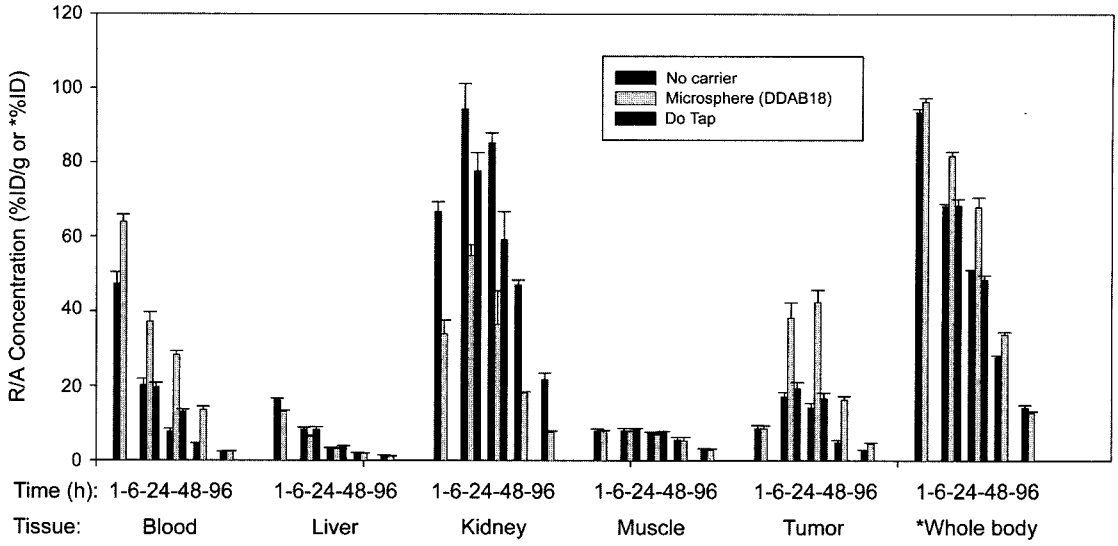

Fig. 1. Biodistribution of [S-35]-labeled MBOs based on the time after injection (n=4-6).

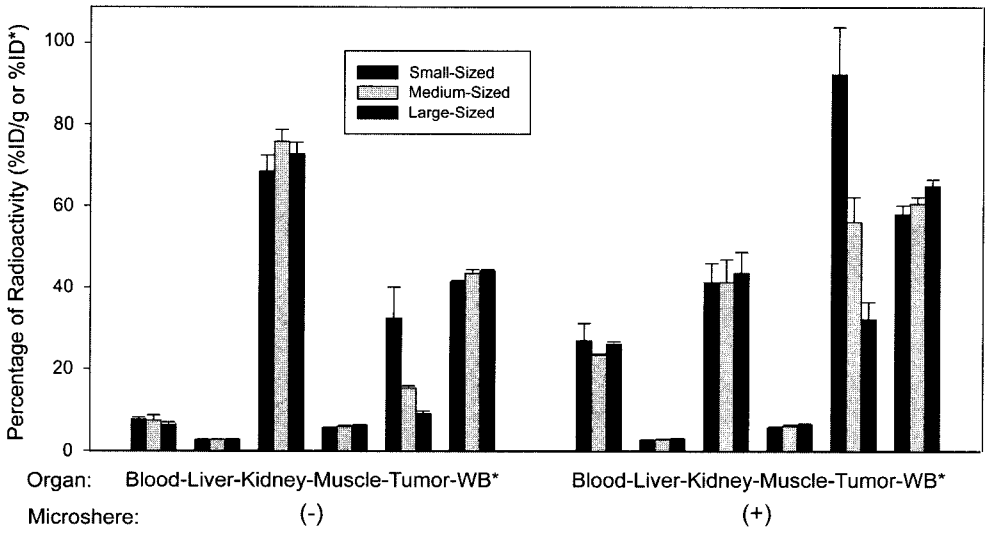

Fig. 2. Biodistribution of [S-35] labeled MB-ON based on tumor-sized variance (n=4).

mg. After 24 hr of the intravenous injection of ODN and microsphere, peak tumor uptake of [S-35]-labeled MBO was significantly increased from 32.4 %ID/g in large-sized to 92.3 %ID/g in small-sized (Fig. 2). Even without microsphere coinjection, this phenomenon was observed in the same pattern: the improvement of uptake from 9.08 %ID/g in large-sized to 32.41 %ID/g int small-sized. Other normal organs or tissues, including blood, liver, kidney, and muscle, were not significantly affected by the size of tumor developed after the subcutaneous injection of cancer cells, except that the whole body retention was slightly decreased depending on the increase of tumor size. Apparently, microshpere coinjection improved the targeting of [S-35]-labeled MBO into the tumor mass xenografted and its impact was extraordinarily significant on the small-sized tumors. The higher susceptibility of [S-35]-labeled MBO in the small-sized tumor can be anticipated for detecting and treating cancer patient from the very early stage of carcinogenesis.

Discussion

This study demonstrates that the presence of the microsphere, a cationic-charged polymer, allows improvement of the

Table 1. Biodistribution of [S-35]-labeled antisense oligodeoxynucleotides based on the time after injection (n = 4-6)*

Time (h)	Microsphere Coinjection	Blood	Liver	Kidney	Muscle	Tumor	Whole body
1	No	47.3±3.2	16.0±0.5	66.7±2.7	7.8±0.6	8.6±0.9	93.5±0.9
6	No	21.5±2.2	8.1±0.3	100.2±5.1	7.8±0.7	17.7±1.3	70.1±1.1
24	No	7.8±0.8	3.2±0.2	85.3±2.7	7.2±0.3	14.1±1.2	50.8±0.3
48	No	4.4±0.3	2.0±0.1	47.1±1.4	5.4±0.8	4.8±1.2	27.7±0.5
96	No	2.3±0.1	1.2±0.3	21.6±1.8	3.0±0.3	2.5±0.3	14.2±0.7
1	Yes	63.9±2.0↑	13.1±0.3↓	34.0±3.6↓	7.8±0.2	8.6±0.8	96.3±1.1
6	Yes	37.2±2.6↑	6.3±0.3↓	54.9±2.9↓	7.7±0.3	38.2±4.1↑	81.8±1.1↑
24	Yes	28.3±1.0↑	3.2±0.2	36.5±8.9↓	6.9±0.3	42.5±3.3↑	68.0±2.7↑
48	Yes	13.5±0.9↑	1.9±0.0	18.2±0.3↓	5.3±1.0	16.2±1.0	33.8±0.7
96	Yes	2.4±0.1	1.1±0.1	7.6±0.3↓	2.9±0.3	4.5±0.3	12.8±0.5

* Percentage of Injected Dose per gram of Tissue (%ID/g) except Whole body (%ID).
↑ (higher) or ↓ (lower) than control (No Microsphere Coinjection) group, $p<0.05$ by One-way ANOVA.

Table 2. Biodistribution of [S-35]-labeled antisense oligonucleotides based on the size of the tumor xenografted (n = 4-6)*

Size (mg)	Microsphere Coinjection	Blood	Liver	Kidney	Muscle	Tumor	Whole body
S (19746)	No	7.6±0.5	2.7±0.2	68.4±4.0	5.7±0.2	32.4±7.6	41.4±0.9
M (41929)	No	7.4±1.2	2.7±0.1	75.8±3.0	5.9±0.3	15.3±0.6↓	43.4±1.0
L (70258)	No	6.4±0.6	2.8±0.2	72.7±2.9	6.2±0.2	9.1±0.6↓	44.0±0.3
S (20719)	Yes	26.9±4.2↑	2.7±0.2	41.3±4.6↓	5.7±0.3	92.3±1.2↑	58.0±2.2↑
M (35534)	Yes	23.4±0.3↑	2.8±0.2	41.4±5.5↓	6.1±0.4	56.1±6.1↑	60.6±1.6↑
L (65670)	Yes	26.1±0.7↑	3.0±0.2	43.5±5.3↓	6.5±0.2	32.4±4.2	64.8±1.7↑

*Percentage of Injected Dose per gram of Tissue (%ID/g) except Whole body (%ID) after 24 h of Intravenous Injection.
↓ (higher) or ↓ (lower) than small-sized control group, $p<0.05$ by One-way ANOVA.

pharmacokinetics of [S-35]-labeled AS-ODN targeted to cAMP-dependent protein kinase (PKA) RI-α subunit in the WiDr (human colon cancer, ATCC CCL218) tumor model of nude mice. This may be explained by the fact that the microsphere plays an important role in an efficient shuttle for the ODN to accumulate increasingly in the cancer cells. With microsphere coinjection, biodistribution of radiolabeled MBO shifted positively in a point of tumor targeting: 1) increase of blood retention; 2) decrease of kidney accumulation; and 3) eventual increase of cancer-cell targeting. One possible hypothesis for the microsphere function is that ODN could incorporate to the microsphere rapidly and is released from it constantly in proportion to the blood concentration. During the early phase of the injection (<6 h), the incorporation of microsphere and ODN decreases the accumulation in the liver and the kidney but increases the blood concentration, leading to a higher concentration of radioactivity in tumor mass at 6 h and 24 h after the injection. In addition, the catabolite of microsphere such as the positively-charged surfactant may affect the kidney accumulation: possibly avoiding glomerular filtration and masking negatively-charged membrane in the proximal tubules. This can lower the nonspecific accumulation of radioactivity in the kidney, leading to the increase of whole-body retention and cancer-cell accumulation.

When the microsphere function is compared with variable sizes of tumors, small-sized (202±33 mg) tumors are more susceptible than medium-sized (387±32 mg) and large-sized (679±64 mg) tumors. Most human solid tumors do not grow with a constant doubling rate. Instead, the rate of growth slows progressively with increasing tumor size. Patients with large tumors often respond poorly to cancer treatment, primarily because of unfavorable tumor cytokinetics. Thus, microsphere coinjection can help produce a higher impact on small-sized tumors, which are in a maximal growth fraction and have favorable tumor cytokinetics, than bigger-sized tumors.

In summary, microsphere coinjection definitely improved the tumor targeting of AS-ODN in the human colon cancer model of nude mice, especially in small-sized cancer masses xenografted. Thus, the microsphere appears to be an important carrier system in vehiculation of antisense oligonucleotide into the tumor tissue *in vivo*.

Acknowledgements

This work was supported by Korean Health 21 R&D Project, Ministry of Health & Welfare (Grant# HMP-00-GN-01-002) and Brain Korea 21 Project, Ministry of

Education, Republic of Korea.

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