Oligonucleotides Targeted Against a Junction Oncogene Are Made Efficient by Nanotechnologies

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Purpose. Antisense oligonucleotides (AON) against junction EWS-Fli-1 oncogene (which is responsible for the Ewing Sarcoma) are particularly interesting for targeting chromosomal translocations that are only found in tumor cells. However, these AON have proved in the past to be ineffective *in vivo* because of their susceptibility to degradation and their poor intracellular penetration. The aim of this study was to improve the delivery of these molecules through the use of nanotechnologies.

Method. Two different AONs, and their controls, both targeted against the junction area of the fusion gene EWS-Fli-1 were used. Nanocapsules were employed to deliver a phosphorothioate AON and its control. The nanospheres were used to deliver a chimeric phosphorothioate, phosphodiester AON, with 5 additional bases in 5′ which allow this AON to be structured with a loop. These formulations were injected intratumorally to nude mice bearing the experimental EWS-Fli-1 tumor. The tumour volume was estimated during the experiments by two perpendicular measurements length (a) and width (b) of the tumour and was calculated as $ab^2/2$. Northern blot analysis was also performed after removing the tumors 24 h after the treatment with a single dose of AON either free or associated with nanotechnologies.

Results. This study shows for the first time that AON against EWS-Fli-1 oncogene may inhibit with high specificity the growth of an EWS-Fli-1 dependent tumor grafted to nude mice provided they are delivered by nanocapsules or nanospheres. In this experience, the antisense effect was confirmed by the specific down regulation of EWS-Fli-1 mRNA.

Conclusion. Thus, both nanocapsules and nanospheres may be considered as promising systems for AON delivery in vivo.

KEY WORDS: nanospheres; nanocapsules; junction oncogene; Ewing sarcoma; antisense oligonucleotides.

INTRODUCTION

The elucidation of the genetic basis of a number of diseases has led to an enormous enthusiasm for newly available proteins and/or nucleic acids as drug candidates. However, many of these compounds have proved to be ineffective *in vivo* because of their susceptibility to degradation and because of their poor ability to gain access to their intracellular targets. This has been the case for antisense oligonucleotides (AON), which, with a few exceptions, have not fulfilled their potential as clinically useful drugs. Our opinion is that more effort should be directed toward tailoring efficient delivery systems for these molecules. This is illustrated by the use of AON against junction oncogenes that are found in cancers such as certain leukemias, Ewing sarcoma, and thyroid papillary carcinomas. These tumors are particularly interesting targets because they originate from a chromosomal translocation which is therefore found only in the tumor cells. However, successful results have never been obtained with AON directed against junction genes on solid tumors *in vivo*, probably because of their short biologic life and limited cellular uptake. We show here for the first time with two different delivery systems that the design of AON that are effective against junction oncogenes *in vivo* is possible, but only when they are delivered to the solid tumor with the aid of nanotechnologies.

MATERIALS AND METHODS

Oligonucleotides

Two different AONs, and their controls, both targeted against the junction area of the fusion gene EWS-Fli-1 were used. Nanocapsules were used to deliver a phosphorothioate AON, and its control, already described by Tanaka *et al.* (1). This AON, called ASp, has the following sequence: 5′-GAG-TGA-GTC-ATA-AGA-AGG-GTT-CTG-C-3′. The sense oligonucleotide, CTp, was used as a control: 5′-GCA-GAA-CCC-TTC-TTA-TGA-CTC-AGT-C-3′. The nanospheres were used to deliver a chimeric phosphorothioate, phosphodiester AON, called ASs, and its control, CTs. ASs has the following sequence: 5′-GTA-GCG-AAG-GGTs-TsCsTs-GsCsTs-GsCC-CGT-AGC-TGC-3′. ASs is complementary to the junction sequence with five additional bases in 5′ that allow this AON to be structured (2) with a loop that is protected by the phosphorothioates. The control, CTs, is also a structured chimeric phosphorothioate, phosphodiester oligonucleotide. Its sequence is: 5′-GTA-GCG-AAG-GGG-GsTsCs-GsTsCs-TsTsC-CCG-TAG-CTG-C-3′. ODN were labeled at their 5′ end using [γ-^{33}P]ATP and T4 polynucleotide kinase.

Nanoparticles

Two types of nanoparticles have been used as carriers of AON: nanospheres and nanocapsules. Nanospheres were prepared by emulsion polymerization and overcoated with cetyltrimethylammonium (CTAB) as previously described (3). Briefly, isohexylcyanoacrylate monomer (IHCA 10 mg/ml) was added to an aqueous solution containing hydrochloric acid (1 mM, pH 3) and dextran 70 (1% w/w). The polymerization occurred spontaneously at room temperature (20°C) under stirring. After neutralization to pH 7, AON (20 μM) complexed with CTAB (50 μM) was added to the suspension of nanospheres for coating.

Nanocapsules were prepared by interfacial polymerization (4,5). In short, 200 μl of demineralized water containing AON at 2.5 mM was added to an organic phase containing 2

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g of Miglyol 812 and 0.25 g of Montane 80. Ten milligrams of IBCA was then added slowly to the emulsion. After 4 h, polymerization was complete. Resuspension of nanocapsules in an aqueous medium was achieved by ultracentrifugation at 37,000 *g* for 30 min in a centrifuge tube filled with 1.5 ml of demineralized water. After removal of the oily phase, the interface and the water phase, the pellet was resuspended in 2.5 ml of demineralized water under vortex agitation (1 min) followed by sonication (30 s).

Diameters of nanospheres and nanocapsules were measured after dilution (1/10) in demineralized water using dynamic laser light scattering (Nanosizer ND4, Coultronics, France). Determination of ODN encapsulation yield was done as follows: radiolabeled ODN were mixed with unlabeled ODN. This isotopic dilution was used to prepare the particles as described above. After ultracentrifugation, radioactivity was counted in Ultima Gold™ with a liquid scintillation system LS6000TA (Beckman, CA) in supernatant and pellet. The final encapsulation yield was calculated using the ratio of the pellet radioactivity to the total radioactivity (pellet + supernatant). For electron microscopic examination, nanosphere or nanocapsule suspensions were frozen in liquid propane and then kept in liquid nitrogen. Fractures and shadowing (Pt/C) were performed in a Balzers BAF freeze-etching unit. The washed replicas were then placed on a copper grid and observed under a transmission electron microscope.

Experimental Tumor

Murine fibroblast NIH-3T3 stably transfected by human EWS-Fli-1 fusion gene (4) were harvested from 60% confluent monolayer cultures, resuspended with PBS at 5×10^6 viable cells/ml, and subcutaneously inoculated into athymic mice (1×10^6 viable cells/mouse). Fourteen days after tumor inoculation, when the subcutaneous tumor had grown to a visible size, different preparations could be injected in the established tumor (day 1). The final oligonucleotide concentration in the nanocapsule or nanosphere suspensions used was 160 μM and 20 μM, respectively. Then, the amount of AON administered in one injection for nanocapsules corresponded to 0.5 mg/kg of AON (eight injections) and to 1 mg/kg for nanospheres (five injections). The complete treatment corresponded to a cumulative dose of 4 mg/kg of ODN for nanocapsules and 5 mg/kg for nanospheres. The tumor volume was estimated during the experiments by two perpendicular measurements—length (a) and width (b) of the tumor—and was calculated as $ab^2/2$.

For Northern blot analysis, tumors were removed 24 h after treatment with a single dose of 2.1 nmol oligonucleotide. For RNA extraction, 600 μl of 4 M guanidium thiocyanate, 25 mM Na citrate, pH 7, 0.5% sarcosyl, and 0.1 M β-mercaptoethanol at 0°C were added to the tumor. The tumor was homogenized with a pellet piston (Eppendorf) and then centrifuged 5 min at 13,000 rpm. RNA extraction with phenol was performed on 400 μl of supernatant. Forty microliters of 2 M Na acetate, pH 4, 400 μl of water-saturated phenol, and 120 μl of chlorophorm:isoamyl alcohol (49:1) was added. The mixture was centrifuged at 13,000 rpm for 15 min, and 300 μl of the aqueous phase was precipitated with 300 μl of isopropanol at −20°C. The pellet was washed with 70% ethanol, dried, and solubilized in 10 μl of water. Electrophoresis of 2 μg of total RNA was performed on a 1% agarose gel in MOPS buffer containing 6.3% formalin. The RNA was then blotted on a nitrocellulose membrane (BA-S85, Schleicher & Shuell) in 10× SSC buffer. EWS-Fli-1 mRNA was detected by the Northern technique with an 800-bp fragment of EWS-Fli-1 cDNA labeled with [α-^{32}P]dCTP, 300 Ci/mM (ICN, France), and nonaprime II random priming kit (Appligen, France). After washing, the nitrocellulose was analyzed with a Storm 840 phosphorimager (Molecular Dynamic).

RESULTS AND DISCUSSION

The morphologies of nanospheres and nanocapsules were clearly different, as shown by transmission electron microscopy after freeze fracture (Fig. 1) Nanospheres are homogeneous nanomatrix systems (100 nm) made of polyisohexylcyanoacrylate and coated with the hydrophobic cation CTAB to allow the binding of negatively charged AONs on their surface, whereas polyisobutylcyanoacrylate nanocapsules (320 nm) are, on the contrary, heterogeneous in size; they consist of a similar polymeric shell surrounding an aqueous core containing the AONs. The yield of AON encapsulation was 81 ± 8% for nanocapsules and 10 ± 2% for nanospheres.

Both types of AON-loaded nanoparticles dramatically inhibited the growth of the xenografted tumor after intratumoral injection into nude mice, whereas similar nanoparticles with control oligonucleotides had no effect (Table I). Free AON was also without effect. With AON in nanospheres, we have shown after 24 h that the mRNA of EWS-Fli-1 was specifically down-regulated, confirming the antisense activity of the targeted AON. Depending on the normalization used (either 18 S RNA or 28 S RNA), the inhibition by the AON

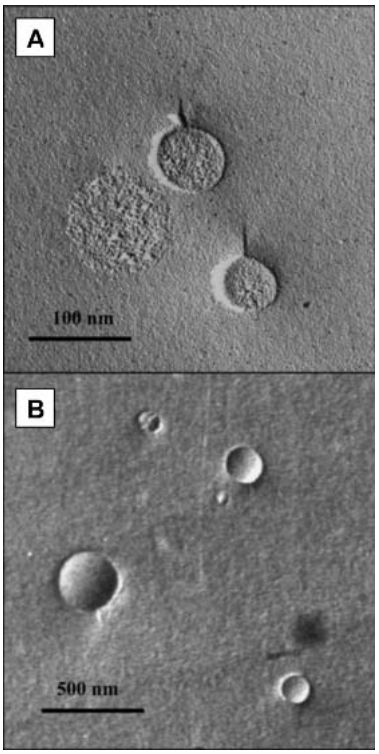

Fig. 1. Electron microscopy after freeze-fracture of (A) nanospheres and (B) nanocapsules.

Table I. Influence of the Type of Carrier on Antisense Oligonucleotide-Induced Inhibition of EWS-Fli-1 Tumor Growth in Nude Mice

Carrier	Oligo	Cumulative oligonucleotide dose (mg/kg)	Day 17 tumor volume ratio (treated/saline)	Inhibition (%)	p-Value*
Nanospheres†	ASs	5	0.18	82	<0.0001
Nanospheres†	CTs	5	0.96	4	0.4217
No carrier†	ASs	5	0.90	10	0.0196
Nanocapsules‡	ASp	4	0.34	66	<0.0001
Nanocapsules‡	Ctp	4	0.97	3	0.6212
No carrier‡	ASp	4	0.77	23	0.0476
No carrier‡	Ctp	4	0.81	19	0.1396

ASs, structured chimeric phosphodiester phosphorothioate antisense oligonucleotide; CTs, structured chimeric phosphodiester phosphorothioate control oligonucleotide; Asp, full phosphorothioate antisense oligonucleotide; Ctp, full phosphorothioate control oligonucleotide.

* The p-values, obtained by analysis of covariance, are those of the comparisons to saline-treated animals of tumor size at day 17 using size at day 1 as covariable and treatments as factors.

† The analysis of covariance, with the size at day 1 as covariable, shows that the nanospheres ASs give an inhibition that is significant when compared to saline-treated animals ($p < 0.0001$), but the nanospheres CTs do not give an inhibition ($p = 0.4217$). Each group contains five animals. The difference between nanocapsules ASp and ASp alone is highly significant ($p < 0.0001$).

‡ The analysis of covariance gives a p-value below 0.0001 for the comparison of nanocapsules Asp to saline. Other p-values are all above 0.05, but for ASp alone, this last value is nevertheless not significant if a Bonferroni correction for test multiplicity is applied.

was 75% or more, and the effect of the control oligonucleotide ranged from 0 to 40% inhibition (Fig. 2).

The mechanism underlying the efficacy of both nanocapsules and nanospheres loaded with AON on experimental Ewing sarcoma needs further investigation. It was suspected that, in the case of nanospheres, the cationic surfactant CTAB could permeabilize the endosomal membrane, thus inducing a more efficient cytoplasmic delivery of AON. However, as shown in this study, nanocapsules, although they didn't contain any cationic surfactant, were as efficient as nanospheres in inhibiting the tumor growth *in vivo*. Current investigations are now trying to ascertain if the cyanoacrylic polymer by itself could be responsible for avoiding AON endosomal or lysosomal degradation.

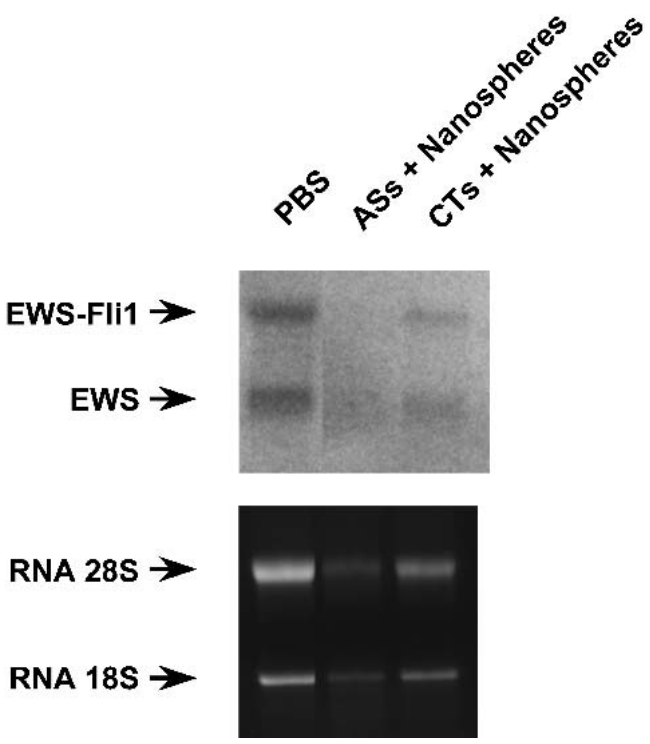

Fig. 2. Inhibition of EWS-Fli-1 mRNA by nanosphere-associated antisense oligonucleotide ASs. CTs oligonucleotide was used as a control.

In conclusion, polyisobutyl- and polyisohexylcyanoacrylate nanoparticles were found to be very efficient systems for AON administration in experimental tumor-bearing chromosomal translocation. It is likely that small interfering RNAs (siRNAs) in mammalian cells, which have recently been described as very powerful gene inhibitors, will require a similar approach for intracellular delivery.

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