방사성동위원소 표지 치료용 올리고뉴클레오티드를 이용한 나노핵폭파 유전자 치료법 소개

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Introduction of Radiolabeled Therapeutic Oligonucleotides As Nanonuclear Explosive Gene Therapy

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Introduction

Since the synthetic oligonucleotide technology has potential application to human diseases and displays promising results in cell-free systems, tissue cultures, and animal models. It is also at early trial points in human testing against HIV, leukemia, Herpes virus, and other diseases, whose outcome will remain for the future. The current status of these varied approaches is presented in later parts in this article:

1) What are therapeutic oligonucleotides?;
2) Why Auger-emitters are useful in Gene Therapy?;
3) What is the synergistic effect on combining Auger emitter and Triplex-forming ODN?;
4) How have TFO researches evolved from the starting point?;
5) In which areas of clinical research will this research illuminating?

Therefore, our first objective in these article was to determine what is therapeutic oligonucleotides and how Auger emitter and TFO combines the synergy on nanonuclear exploasive therapy.

1. What are therapeutic oligonucleotides?

Therapeutic oligonucleotide(s) is defined as a short-sequenced polymer of a few (2-20) synthesized nucleotides pertaining to therapeutics or to therapy including triplex-forming oligonucleotides (TFO), transcription factor decoy, antisense oligonucleotides (ASO), ribozymes, peptide nucleic acids (PNA), 2',5'-oligoadenylates, CpG immune stimulating oligomers, diagnostic oligonucleotide chips and gene vectors. These oligonucleotides can modulate gene-specific expression within cells, and can identify genes involved in diseases. It is generally classified as DNA-

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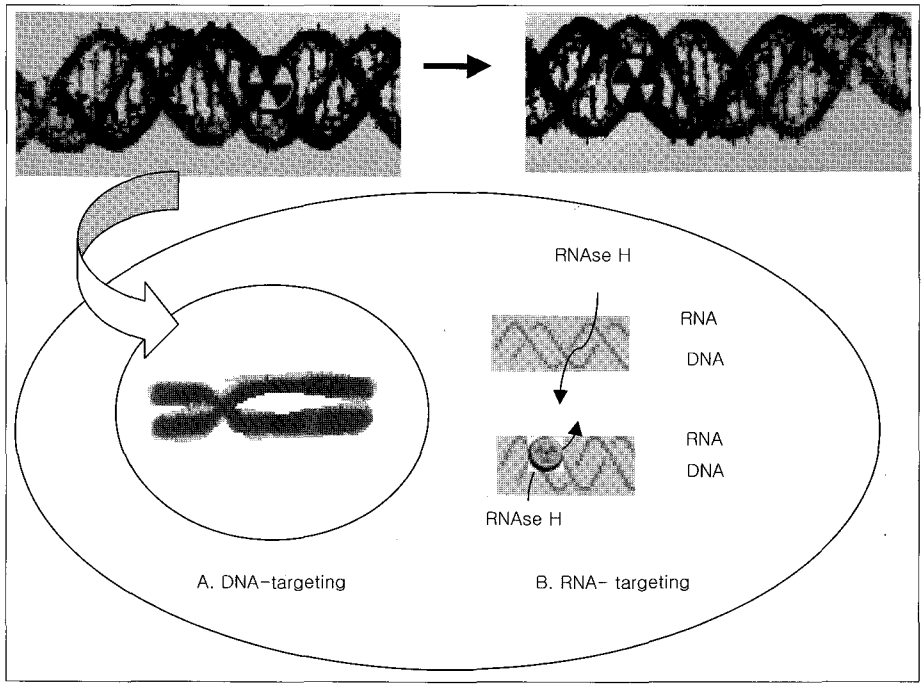

Fig. 1. Scheme of Nanonuclear Explosive Gene Therapy Using Therapeutic Oligonucleotides. A. Triplex- forming oligonucleotide carrying Auger electron emitter binds in the major groove of the target duplex sequence. Decay of Auger electron-emitting radionuclide produces DNA strand breaks within five nucleotides from the decay site. B. Antisense oligonucleotide carrying Auger electron emitter also has a capability to potentiate RNase H function at the cytoplasm.

targeting (e.g., TFO) and RNA-targeting (e.g., ASO) therapeutic oligonucleotide(s) in accordance with the targeting nucleotides as shown in Fig. 1.

In a DNA triplex, the triplex-forming oligonucleotide (TFO), a short oligonucleotide generally 15-20 base pairs (bp) in length, incorporate themselves into the major groove of DNA double helix in a sequence-specific fashion. Hoogsteen bonds are formed with the purines of the Watson-Crick base pairs in a sequence-specific fashion. The existence of TFO structures was first demonstrated in 1957, at the National Institutes of Health (NIH) in the United States by Gary Felsenfeld, David Davies, and Alex Rich. In general, stable triplexes can be formed between polypurine-polypyrimidine duplexes and polypurine or polypyrimidine TFOs. Such sequences are widespread in eukaryotic genomes and are often found in regulatory regions. We have shown the TFOs can serve as suitable vehicles to deliver iodine-125 (^{125}I) or indium-111 (^{111}In) to a specific sequence in a DNA target of interest.

Antisense oligonucleotide (ASO) represents a new paradigm for drug discovery that holds great promise to deliver potent and specific drugs with fewer undesired side effects. It offers the opportunity to identify rapidly lead compounds based on knowledge of the biology of a disease process, and a relevant target gene sequence. Among several hypotheses for the basic mechanism of ASO as a therapeutic strategy, a hypothesis of specific digestion of DNA/RNA hybrids by RNase H is dominant so that the radioisotope labeling of ASO can potentiate the

blockade of RNA expression to protein not only by RNase H but also by radiodecay. Although the ASO paradigm holds great promise, the field is still in its early stages, and there are a number of key questions that need to be answered and technical hurdles that must be overcome, including pharmacokinetic, pharmacological, and toxicological properties. Thus, we would emphasize the current status of research improvement on TFO in the following parts of this review article.

2. Why Auger-emitters are useful in Gene Therapy?

The radiodecay of Auger emitters produces a cascade of low-energy electrons, named after Pierre Auger, who first described this process in 1929. For example, radiodecay of ^{125}I results in the emission of approximately 20 electrons of varying energy. Most of these Auger electrons have initial energies of less than 1 keV and a maximum range of only a few nanometers.

To demonstrate TFO binding by radiolabeled TFOs, we devised a plasmid model system in which we showed DNA double-strand breaks could be produced by TFOs carrying radioactivity. Then, we measured the frequency and distribution of the breaks to show the small ($\pm$ 5 bp) zone of DNA damaged by Auger electrons emitted during radioactivity decay. Double-strand breaks produced by decay of Auger-emitter incorporated into genomic DNA are highly radiotoxic and hardly reparable. Their repair usually results in deletion of large (>100 kbp) fragments of DNA. Radiolabeled TFOs which do not form triplexes with genomic sequences, yet are present in the nucleus, caused very little radiation damage. These nonbound oligos are nearly 1/300 as toxic to the cell as ^{125}I-5-iododeoxyuridine, a precursor of DNA synthesis that is incorporated into genomic DNA. This gives us a hope that we can produce breaks in targeted gene sequences without causing excessive non-specific radiation damage.

3. What is the synergistic effect on combining Auger emitter and Triplex-forming ODN?

Triplex-forming oligonucleotides labeled with radionuclides that are Auger electron emitters could prove to be ideal vehicles for delivering radioactive decay energy to specific DNA sequences, causing local DNA breaks and subsequent inactivation of genes containing the target sequences. The radiodecay of incorporated ^{125}I from a TFO in a triplex structure with a targeted sequence in duplex DNA produces strand breaks located within 10 bp of the decay site with an efficiency close to one break per decay. The half-life of phosphodiester TFO in cell culture is hours and even shorter *in vivo*. For this reason, we have to freeze the cells after TFO delivery to accumulate DNA breaks and are now developing labeling procedures for phosphoramidate TFOs that are considerably more stable *in vivo*. Of course, for the therapeutic application we will need radioisotopes with a shorter half-life than ^{125}I. Therapeutic applications of Auger electron emitters depend on developing methods for radionuclide delivery to the intranuclear genome of target cells, for example, cancer cells or perhaps even virally infected cells. The promise of TFOs carrying Auger electron-emitting radionucleotides may be gene-specific radioaction therapy if the complexities of triplex formation *in vivo* can be resolved.

4. How have TFO research methodologies evolved from the starting point?

We have shown that TFOs can serve as suitable vehicles to deliver ^{125}I to a specific

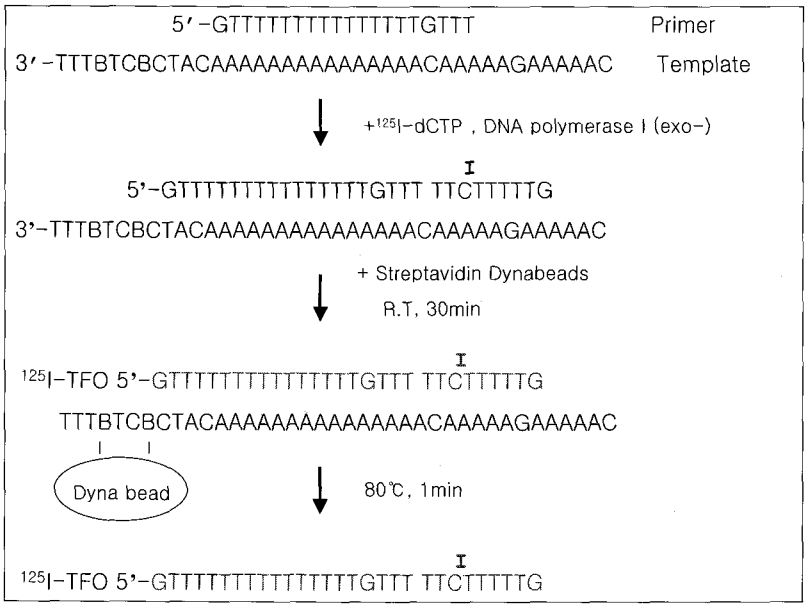

Fig. 2. Synthesis of ^{125}I labeled TFO. Two synthetic oligonucleotide, primer and template, which is biotinylated at the 3′-end, were annealed and primer extension is done at the 3′-end of the primer by DNA polymerase I (Klenow fragment). The ^{125}I TFO were purified by denaturing the resulting duplex and separated by precipitation of the biotinylated template.

sequence in a DNA target. The decay of ^{125}I incorporated into a TFO as an iododeoxycytidine residue produces double-strand breaks in the target duplex on triplex formation *in vitro* with an efficiency close to 1 break per decay. The decay of ^{125}I was found to cause double-strand breaks within the cancer gene upon triplex formation in a sequence radiotherapy is our approach to targeting specific sites in the genome by combining the highly localized DNA damage produced by the decay of Auger electron emitters, such as ^{125}I, with the sequence-specific action of TFO. Two DNA sequences (39-bp and 36-bp, respectively) of human ER gene promoter (hERP) were chosen as the target sites for triplex formation. PCR product containing polypurine-polypyrimidine elements was subcloned using primers amplifying 376-1144 (769-bp, hERP1) and 3601-3807 (207-bp, hERP2).

The phosphodiester TFO was labeled with ^{125}I-dCTP at the C5 position of cytosine (marked with star in Fig. 2) by the primer extension method. Gel filtration through a Sephadex G-50 MicroSpin column revealed that 199 μCi ^{125}I was incorporated into the duplex. Gel-shift analysis of the binding is demonstrated in Fig 3. By measuring the intensity of the bands corresponding to the free [^{125}I] TFO and the triplex we estimate that approximately half (55-64%) of the radioactivity was associated with the triplex band, which appeared as a band comigrating, with the plasmid ('bound'). As expected, no binding occurred in the 'not complementary' controls.

The human malignant melanoma cell line (SK-MEL1; ATCC Number HTB-67), Ductal carcinoma cell line (T-47D;ATCC Number HTB-133) was grown in monolayer in RPMI 1640 Medium, supplemented with 0.1 mM non-essential amino acids and 10% fetal bovine serum (FBS), purchased from BinWhittaker

(Walkersville, MD). All procedures were done according to ATCC recommendations. Cells grown to confluence were treated with trypsin, dispensed into green tubes. Collected by centrifugation at 1,000 rpm for 5 min and resuspended in 1ml of RPMI 1640 medium to a final concentration of 5×10^6cells/ml. Thirty 800 μl and six 750 μl aliquots were prepared, 20 uCi of the [^{125}I] TFO complex were added to the cells in each of thirty tubes and the tubes were place into a CO_2 incubator. The radioactivity in the cell pellet and the supermant medium was counted in a gamma scintillation counter.

To assess the intracellular distribution of decays, silver grains were counted in 30 randomly selected cells. The mean numbers of grains found in nucleus and cytoplasm were calculated and used to assess the dose delivered by the labeled compounds into cells (Fig. 4).

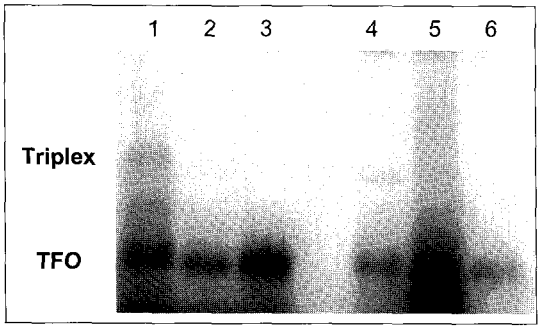

Fig. 3. Triplex formation could be detected by electrophoresis and autoradiography. Lane 1, n-myc dsDNA + n-myc TFO; Lane 2, ER dsDNA + n-myc TFO; Lane 3, n-myc TFO only; Lane 4, ER dsDNA + ER TFO; Lane 5, ER dsDNA + n-myc TFO; Lane 6, ER TFO only.

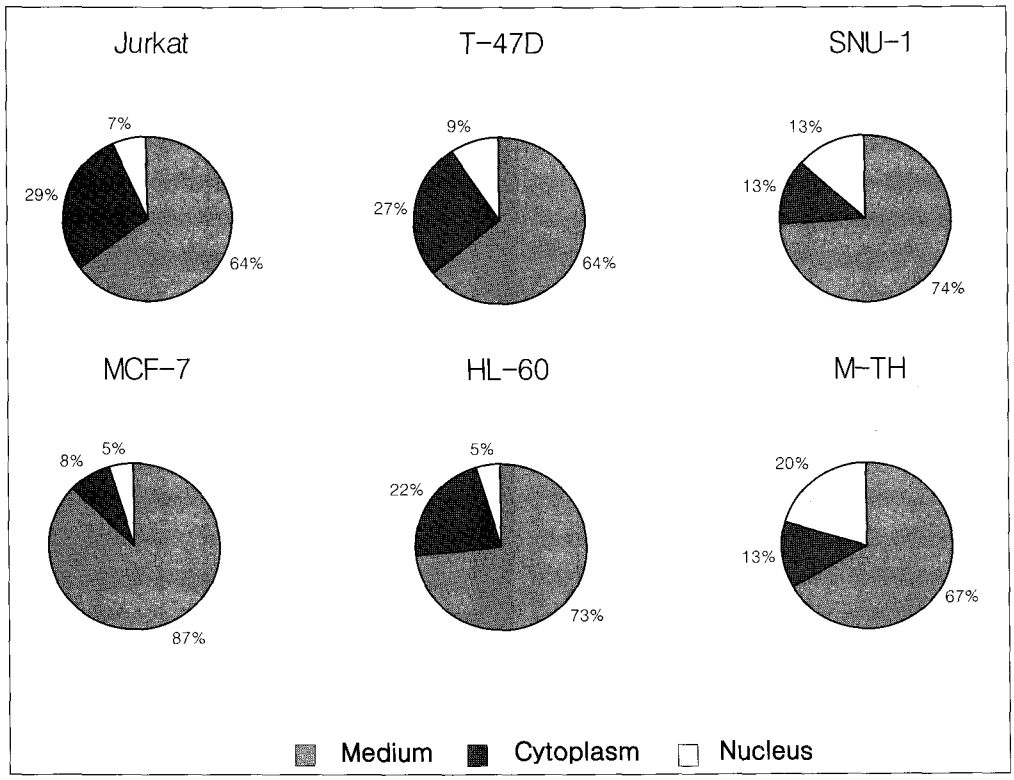

Fig. 4. Subcellular localization of Radiobeled TFOs in various cancer cells

5. In which areas of clinical research will this research illuminating?

in vivo experiments showed that both these two target sites are able to form triplex with their corresponding oligonucleotides. Clinically, treating diseases such as cancers containing amplified genes or viral-derived foreign sequences may be the best application of this technique should it be proven to work *in vivo*. One of the most frequenct difficulties of in hormone (anti-estrogen) therapy in breast cancer is that almost all breast cancer progress to a hormone-resistant state. Estrogen receptor (ER) status is used clinically both as a prognostic factor and as a target in the therapy of breast cancer. Some investigators have found that the upstream promoter is utilized only in breast cancer cell lines that express high levels of ER and not in primary human mammary epithelial cells. Normal breast epithelial cells show a very low level of ER expression, thus raising the possibility that the very high level of ER expression in some tumors is secondary to the function of a trans-acting factor not active in normal breast epithelium.

For the NIH basic scientist, TFO method may be useful to probe nucleic acid-protein complexes because the Auger electron damage is so focal and is distance-related. At the NIH (e.g., NCI and NIDDK), Igor Panyutin and collaboratores took this methodological approach when they analyzed decay-induced DNA breaks to successfully examine nucleic acid conformations. Thus, It is also highly likely that clinical research in testing as therapeutic modalities against HIV, leukemia, Herpes virus, and other diseases in near future.

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