Antisense and Nuclear Medicine

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Despite many uncertainties concerning mechanism, synthetic single-strand antisense deoxyribonucleic acids (DNAs) are now in clinical trials for the chemotherapy of viral infections such as human immunodeficiency virus (HIV) and human papilloma virus; several cancers, including follicular lymphoma and acute myelogenous leukemia; inflammatory processes such as Crohn's disease and rheumatoid arthritis and in allergic disorders. There are approximately 10 trials, and early results are generally encouraging. Therefore, the expectation is that antisense DNAs will be important to future chemotherapy. The question considered here is whether antisense DNAs will also be important to future nuclear medicine imaging. While efforts toward developing antisense imaging are comparatively nonexistent thus far, investigations into the mechanisms of cellular transport and localization and the development of a second generation of antisense DNAs have occurred largely within the antisense chemotherapy industry. Fortunately, many of the properties of DNA for antisense imaging, such as high in vivo stability and adequate cell membrane transport, are the same as those for antisense chemotherapy. Unfortunately, interests diverge in the case of several other key properties. For example, rapid localization and clearance kinetics of the radiolabel and prolonged retention in the target are requirements unique to nuclear medicine. No doubt the development of antisense imaging will continue to benefit from improvements in the antisense chemotherapy industry. However, a considerable effort will be required to optimize this approach for imaging (and radiotherapy). The potential of specifically targeting virtually any disease or normal tissue should make this effort worthwhile.

Key Words: antisense; radiolabeling; deoxyribonucleic acid; imaging

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Antisense chemotherapy may be defined broadly as the treatment of disease through the administration of synthetic single-strand oligodeoxyribonucleic acids (DNAs) or oligoribonucleic acids (RNAs) designed to bind in a sequence-specific manner to the machinery of translation or transcription and thus interfere by some antisense mechanism with the expression of a key gene and the production of its protein. Since antisense chemotherapy was first proposed (*1,2*), attitudes have roller-coastered from initially optimistic to quite negative, as the many complexities and difficulties of the approach became apparent (*3,4*). Recent attitudes, however, are again positive, as the mechanisms of action become better understood and as potential solutions to the many difficulties are proposed (*3,5*).

Adding to the upbeat mood are the generally encouraging results being reported in clinical trials of antisense chemotherapy. Antisense DNAs are under investigation for the treatment of viral infections (*6*), various cancers (*7*) and inflammatory disorders (*8*). Approximately 10 different antisense DNAs are now in clinical trials (*9*). An antisense DNA that blocks replication of cytomegalovirus has recently been approved by the U.S. Food and Drug Administration for the treatment of patients with acquired immunodeficiency syndrome; Phase II trials of another antisense DNA for the treatment of Crohn's disease are showing remission in nearly half of patients treated; and researchers are reporting that another antisense DNA shrinks ovarian tumors (*3*). Results are not uniform. Two antisense DNAs, one against genital warts and another against human papilloma virus, were discontinued in Phase II. The efficacy of antisense chemotherapy has been reviewed (*10,11*). Antisense is also becoming useful as a research tool in molecular biology (*12*).

All current clinical trials of antisense chemotherapy use uniformly modified phosphoromonothioate DNAs in place of the native phosphodiester DNAs (*13*). The success now being achieved with antisense chemotherapy may be attributed in large part to the development of this chemically modified DNA. The chemical structures of uniform phosphodiester DNA, phosphorothioate DNA and methylphosphonate DNA, another useful modification, are shown in Figure 1. The phosphorothioate and (to a much lesser extent) the methyphosphonate derivatives are still the most popular of the hundreds of DNA derivatives that have been synthesized (*14–16*). In both cases, one nonbonding oxygen atom of each phosphate (in the uniformly modified DNAs) has been replaced with either a sulfur (phosphorothioate) or methyl group (methylphosphonate). Both changes greatly improve the in vivo stability of the DNA relative to the phosphodiester DNA (see below) and provide the rationale for their use. Both modifications also introduce a chiral center on each phosphate, whereas only the phosphorothioate modification preserves the negative charge of the phosphodiester DNA. The structure of RNA is essentially identical to DNA, with the addition of a chemical group replacing the hydrogen in

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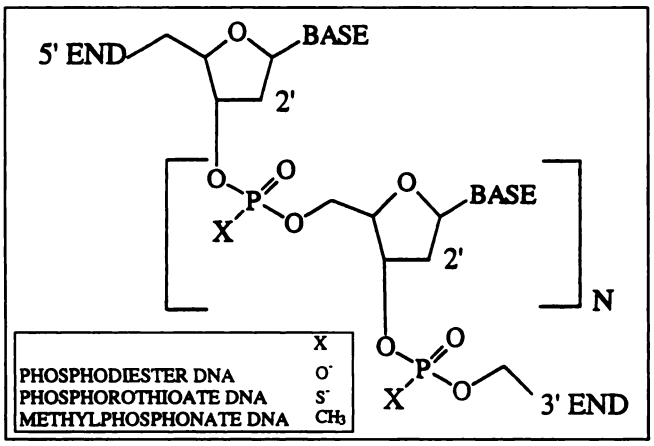

FIGURE 1. Chemical structures of phosphodiester DNA (X $=O^-$), phosphorothioate DNA (X $=S^-$), and methylphosphonate DNA (X $=CH_3$).

the 2′ position of the deoxyribose sugar and the substitution of a uracil base for every thymine.

From the point of view of radiopharmaceutical development, as well as drug development, DNAs are remarkable molecules. Through their property of hybridization, a single-strand DNA can display extreme affinity for its complementary single-strand DNA. For example, an 11-base phosphodiester DNA can hybridize with picomolar affinities, depending on base sequence and chemical environment (*17*). DNAs also display extreme specificities. In the above case, introducing 2 central mismatched bases reduces the affinity 10,000-fold (*17*). In the development of new radiopharmaceuticals, high specificity and high affinity of binding have always been recognized as useful properties. Accordingly, several potential applications of radiolabeled DNA as radiopharmaceuticals have been suggested (*18,19*). One nuclear medicine application, now obvious, involves the localization of radioactivity for imaging in tissues targeted by antisense mechanisms (i.e., antisense imaging).

Relevant to antisense imaging is the considerable effort underway, almost exclusively in industry, to develop antisense chemotherapy. The rationale behind the latter effort is the potential for the treatment of the many diseases involving gene-expression. From the nuclear medicine point of view, the rationale is the potential for specific targeting of these diseases with radioactivity for imaging and, possibly, for radiotherapy (*20,21*).

ANTISENSE MECHANISMS

The mechanisms of antisense chemotherapy are complex and, when broadly defined, can include both transcriptional and translational arrest (*9*). The diagram presented in Figure 2 greatly simplifies these processes. During transcription, the genomic DNA double-helix coding for the gene to be transcribed is uncoiled to allow copying into one (or multiple) pre-messenger RNAs (mRNAs), which are single-strand RNAs complementary to the transcribed genomic DNA. In eukaryotic cells, the pre-mRNA strand is then shortened by the excision of segments (introns) not required for translation; a long series of adenosine bases (poly-A tail) is added to the 3′ end; and several nucleotides on the 5′ end are methylated (capped). The polyadenylation stabilizes the mature mRNA and may play other roles. Capping, also important for mRNA stability, may be important for transport of mRNAs out of the nucleus (*22*). In the cytoplasm, the mRNA is degraded within minutes to hours, but possibly not before being translated one or more times within ribosome

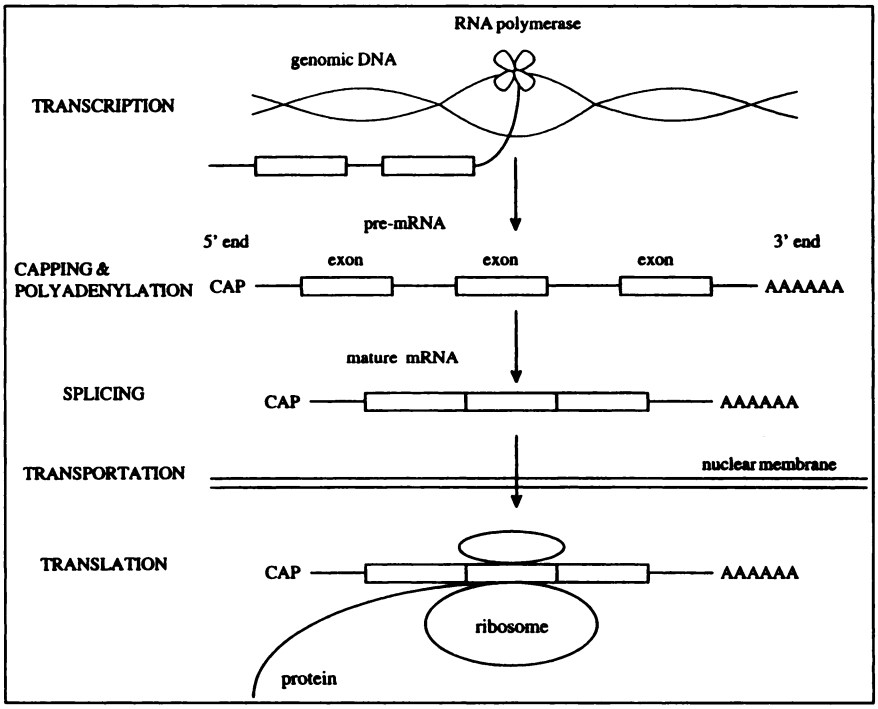

FIGURE 2. Line drawing illustrates transcription of genomic DNA to pre-mRNA, conversion of pre-mRNA to mature mRNA by intron excision, 5′ end capping and 3′ polyadenylation, transport of mRNA out of nucleus and translation to protein within ribosomes [adapted from (*22*)].

organelles into copies of the protein coded by the gene in question (*23*).

Transcriptional Arrest

If the object is to interfere with the expression of a gene (for example, to suppress a mutated protooncogene responsible for unregulated cell division), Figure 2 makes clear that there are obvious opportunities to do so. Various antisense strategies for transcriptional arrest have been suggested for interference with polyadenylation, capping and intron splicing (*6,24*). Transcriptional arrest might follow the addition into the nucleus of a single-strand DNA capable of triple-helix formation (Hoogsteen binding) with the gene in question in a sequence-specific manner (*25*). This binding could interfere with transcription in several ways, for example, by preventing the synthesis of pre-mRNA by RNA polymerase. Another approach (in this case "sense") is the use of double-strand DNAs with base sequences identical to that of the promoter sequence of the target gene to mimick promotion by acting as a decoy (*26*).

Transcriptional arrest (occasionally considered antigene rather than antisense) is receiving limited attention for a variety of reasons. The process requires the antisense DNA to cross not only the cell membrane to gain access to the transcription machinery, but the nuclear membrane as well. Access to the binding sites may also be restricted within the chromatin of the genomic DNA. The rules governing triple-helix formation are not nearly as well understood as those of the Watson-Crick double-helix formation, so that selecting a suitable antisense DNA sequence can be difficult (*27*). In addition, triple-helix formation is not applicable to all genes (*28*). Finally, there is a general unwillingness at present to monkey with the genome for fear of unknown genotoxicities. However, the chemotherapeutic advantage of interfering with the process early (i.e., transcription rather than translation) lies in its efficiency, since a single gene (or a small number) is all that needs targeting at this stage, compared to multiple mRNA copies needed later (*28*).

From the imaging point of view, transcriptional targeting offers all these disadvantages without concomitant advantages. The presence of multiple copies of mRNA provides multiple targets for localization, and so is an advantage for imaging even while being a disadvantage for chemotherapy.

Translational Arrest

Conceptually at least, translational arrest, like transcriptional arrest, is deceptively simple. A single-strand antisense DNA within the cytoplasm may hybridize to the target mRNA in a way that prevents translation. Originally, it was thought that translational arrest always resulted from interference at the ribosome level (*29*). However, it is now recognized that the formation of a DNA/mRNA heteroduplex may not always hinder translation of the mRNA (*30*). Rather, translational arrest is often dependent on ribonuclease H (RNase H) enzymes, which recognize the DNA/mRNA duplex and degrade the mRNA (*31*). If so, this is fortunate, since the antisense DNA then acts as a catalyst and is eventually released to begin the process anew. However, many of the chemically modified DNAs are not substrates for these enzymes and so cannot interfere with translation by this mechanism. For example, the methylphosphonate DNAs do not activate RNase H (*15*). The phosphorothioate DNAs are potent antisense inhibitors, possibly because they are substrates for RNase H (*6*).

Figure 3 illustrates a complexity of translational arrest. The figure diagrams the two-dimensional structure of an mRNA. Rather than the single-strand mRNA that might be expected, the molecule has a complex secondary structure with many regions of intrachain base pairing. In contrast to single-strand DNAs, single-strand RNAs form extensive secondary structures (*30*). The secondary structures of mRNA may be required for stability and possibly for recognition by those proteins regulating translation. These structures have implications for chemotherapy, since antisense DNAs show lower affinities for duplex regions. The

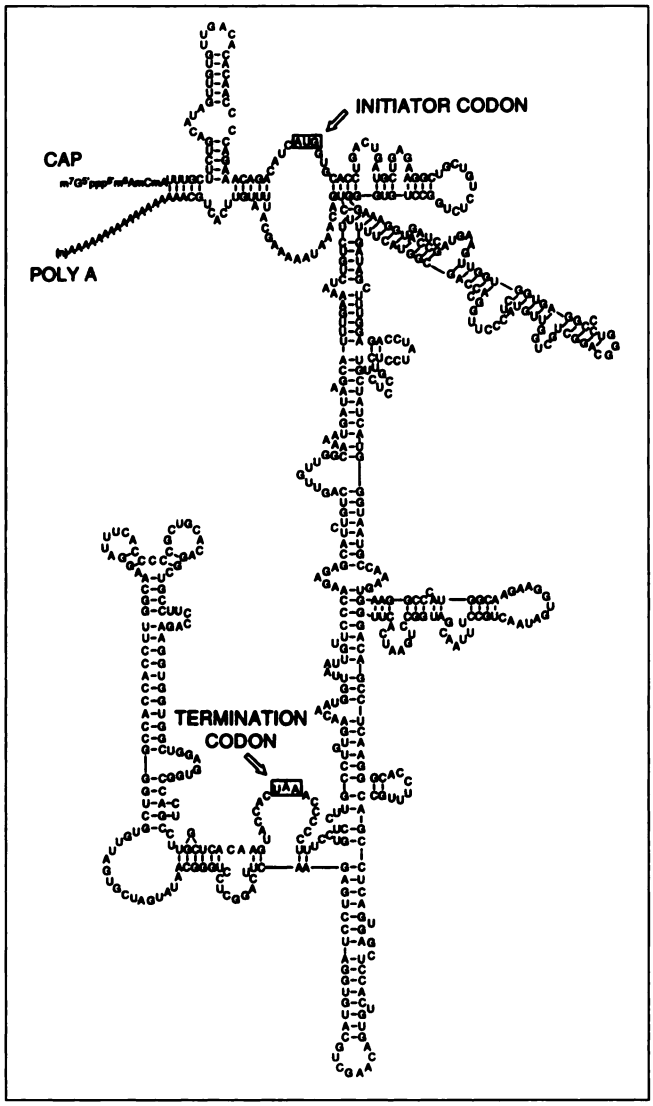

FIGURE 3. Structure of mRNA [Reprinted with permission of (*30*)].

affinity of antisense DNA for duplex, compared to singlet, regions of mRNA has been reported to be 10^5–10^6-fold lower (*32*). Therefore, antisense strategies usually seek to target only single-strand regions of the mRNA. Since knowledge of the molecular structures of mRNAs is limited (*22,33*), one common approach is to target either the initiation codon (AUG) and adjacent sequences or the untranslated sequences on either the 5′ or 3′ end, in the hope that these regions will be accessible (*6,34*).

One final, and important, mechanism of antisense chemotherapy involves ribozymes. Ribozymes are antisense single-strand RNA enzymes which can catalyze the cleavage of specific mRNA sequences (*35*). The second generation of antisense DNAs are largely chimeras, composed of stretches of DNA with gaps of RNA (*29*). The central RNA can act as a ribozyme specific for the targeted mRNA, while flanked by wings of antisense DNA selected to affix the ribozyme appropriately to the mRNA.

The foregoing was intended to introduce antisense mechanisms of action. What follows is a discussion of the concerns and difficulties of mRNA-targeted antisense, including those common to antisense chemotherapy and nuclear medicine imaging and those unique to the latter.

ANTISENSE CONCERNS

Cell Membrane Transport

To achieve therapy or imaging, antisense DNAs must cross the cell membrane and enter the cytoplasm without encapsulation and permanent entrapment in endosomal or lysosomal vesicles. Generally, only a small percentage of DNAs incubated with cells are incorporated under the most favorable circumstances (*36,37*). An open question is whether transport is active or passive. The highly-charged backbone of the phosphodiester and phosphorothioate DNAs probably eliminate any possibility of passive transport. However, even uncharged DNAs are reported to be unable to enter cells by passive diffusion alone (*11,24*). Both the uncharged methylphosphonate DNAs and the uncharged peptide nucleic acids (PNAs) have been reported to show poor-to-nonexistent transport (*38–40*). At present, there is no evidence of a specific receptor for antisense DNAs. Internalization probably follows nonspecific binding to one or more cell-surface proteins (*41*). Active transport can occur, at least for some cell lines and some chemical forms of DNA, after receptor-mediated adsorptive endocytosis or pinocytosis (*42–44*). One argument in favor of an active transport mechanism is the further reduction in cellular uptake at lower temperatures (*45*). While poor cell membrane transport has been clearly demonstrated in vitro (*46*), there is some indication that transport in vivo may be superior (*3*).

The inefficient intracellular localization of antisense phosphorothioate DNAs explains, in part, the large dosages (eg, 0.05 mg/kg/h over 10 d) now being administered to patients in connection with antisense chemotherapy (*47*). Reasonable drug costs and the absence of dosage-limiting toxicities make feasible the administration of gram quantities of these antisense DNAs. For imaging, the problem of inefficient cellular transport may not be so easily resolved, since simply increasing the dosage of radiolabeled DNAs could decrease target/nontarget radioactivity ratios should the excess labeled DNAs accumulate in normal tissues or show delayed clearance from target tissues.

Several strategies are under consideration to improve transport (*48*). Two examples are the co-administration of free or covalently-coupled polycations, such as polylysine, to reduce the negative charge on polyanionic DNAs (*49*) and the use of liposomes as carriers, alone or coated with polyethylene glycol, to increase circulation time (*50,51*). Several of these approaches may be complicated by serum instabilities and toxicities. Approaches to intracellular delivery of DNAs in connection with gene therapy, such as adenoviruses vectors, may someday be adapted to antisense (*52,53*).

It is hoped that the problem of poor cellular transport will soon be resolved, thereby removing perhaps the biggest hurdle to progress in antisense chemotherapy and, especially, to the development of antisense imaging.

In Vivo Instabilities

Regardless of the application, antisense DNAs must survive in plasma and in the cytosol long enough to locate and bind to their target. The main source of in vivo instability of native phosphodiester DNAs, especially single-strand DNAs, is enzyme degradation. Exo- and endonucleases (which attack DNA from its ends and interior regions, respectively) are ubiquitous and are responsible for the rapid in vivo degradation of single-strand phosphodiester DNAs (*54*). The main incentive for chemical modification of the phosphodiester DNA was to improve stability against these nucleases. Fortunately, it appears that virtually any modification to the phosphate backbone (the site of nuclease degradation) will improve stability. Thus, the uniformly modified phosphorothioate DNAs are stable and have become the modification of choice for ongoing clinical antisense chemotherapy trials. However, along with methylphosphonate-modified DNA and others, these first-generation antisense DNAs are far from ideal in other respects, including solubility, chirality, protein binding, cell membrane transport and pharmacokinetics. The second generation of chemically modified DNAs are generally chimeras, consisting of regions of DNA with gaps made of RNA (2′ modified) (*55,56*). The RNA modifications are intended, in part, to provide stabilities against nucleases while improving the affinity of hybridization (*57,58*). However, 2′ modified oligonucleotides are not substrates for RNase H (*59*).

The search for the "perfect" antisense DNA continues.

Antisense Mechanisms

The inhibition of gene expression by mechanisms consistent with antisense has been demonstrated clearly in several investigations in tissue culture. Levels of the target mRNA or target protein have been shown to decrease in a dosage

dependent manner with antisense DNAs, but not with sense or nonsense DNAs (*22*).

Among the chemically modified DNAs, a positive correlation between potency for antisense inhibition and ability to act as a substrate for RNase H suggests that RNase H-mediated translational arrest is a major mechanism of antisense chemotherapy. However, understanding of the exact methods whereby antisense DNAs act, especially in vivo, is far from complete. Few studies have demonstrated that antisense DNAs actually bind to their target mRNA sequences (*59*). The mechanism is likely to be complex, with both sequence-specific and sequence-independent components, especially in the case of DNAs such as the phosphorothioates, which bind to intracellular proteins. Although an understanding of mechanisms may be considered less important in the face of efficacy, studies to establish the exact manner in which the antisense DNAs act continue in the hope of speeding the development of even more effective antisense drugs.

The mechanisms of antisense action are also important for imaging applications, but for different reasons. For example, all current clinical trials of antisense chemotherapy use phosphorothioate DNAs, whereas the use of methylphosphonate DNAs has been studied only in a relatively small number of animal investigations. This is because only the former is a potent antisense inhibitor and a substrate for RNase H. However, for antisense imaging, whether a particular chemical form of DNA is a substrate for RNase H or even a potent antisense inhibitor may be irrelevant to retention of the radiolabel at its specific site. In this example, if the methylphosphonate DNAs display more favorable pharmacokinetics, this chemical form may be more attractive for antisense imaging than the phosphorothioate DNA chemical form (*60*).

In summary, while of critical concern for antisense chemotherapy, the question of whether a particular antisense DNA is a substrate for RNase H or acts as a ribozyme may be important for antisense imaging only to the extent of providing specific binding to the mRNA target. Thereafter, the concern for imaging is the fate of the radiolabel as distinct from the antisense DNA. The more important question is whether the mechanism of action will lead to long-term retention of the radiolabel in the target. It is entirely possible that the antisense mechanisms that provide optimum target localization of a radiolabel will prove to be different from the those that provide optimum chemotherapy. Indeed, since potential collateral damage with potent antisense chemotherapy drugs is still a concern, an antisense sequence with no therapeutic potency may be preferred for imaging (*61*).

Binding Affinity

The affinity of binding of a single-strand DNA to its complement is usually reported as a melting temperature (T_m), the temperature at which half the duplex has dissociated into single strands. Affinity depends on several factors, including the sequence, the chain length and the chemical form of the DNA. For antisense localization, the sequence is dictated in part by the sequence of the mRNA target and by the need to avoid self-hybridization. The chain length is also restricted. To eliminate any statistical possibility of an unintentional complete match with the genome (assuming the genomic base sequence to be random), only about 15–17 bases (approximately 5000 Da) are required (*62*). Lengthening will improve the affinity, but at the possible expense of reducing cell membrane transport even further and, paradoxically, can lower specificity, as small regions find unintended matches (*61,63*). Cost increases, too, with increasing chain length, a factor perhaps of more concern for antisense chemotherapy than imaging. Conversely, shorter strands might not show the required specificity and might reduce T_m values to less than 37°C. Too short a strand will also increase the possibility of unintentional matches with the genome, possibly resulting in collateral damage, already a serious concern of antisense chemotherapy (*30*). Concerning the chemical form, both phosphorothioate and methylphosphonate DNAs are reported to show somewhat lower binding affinities than the phosphodiester DNA, possibly because both modified DNAs are used curently as racemic mixtures (*64*). Nevertheless, the T_m values for a 17-base phosphorothioate DNA hybridized to mRNA should be reduced only about 9°C from that of the phosphodiester and should remain comfortably above 37°C (*59*). In short, adequate affinities should not be a concern for antisense imaging.

Protein Binding

Among the chemically modified DNAs, the phosphorothioate DNAs may be unique in displaying a nonspecific affinity for proteins, possibly through increased lipophilicity, resulting from the presence of sulfur atoms in the backbone (*23*). Binding in serum is primarily to serum albumin, but at low affinities. The binding is also saturable. Fifteen to 20 mg/kg administered intravenously will saturate the serum protein binding capacity in rats (*59*). Along with the need to increase cell membrane transport, the need to saturate tissue proteins is an additional reason for the large dosages of phosphorothioate DNAs administered in ongoing clinical trials. By saturating nonspecific binding sites, the hope is that sufficient DNA will be released for target localization.

The binding of phosphorothioate DNAs to serum proteins may be advantageous for antisense chemotherapy by providing a reservoir for the drug. However, binding has other, less favorable, consequences. Early investigations were misled into ascribing antisense mechanisms to what was ultimately shown to be nonantisense, nonspecific inhibition of proteins due to phosphorothioate DNA protein binding. For example, depending on concentration, phosphorothioate DNAs can interfere with gene expression by inhibiting DNA polymerase or RNase H and also can bind to transcription factors (*65,66*). Early investigators were surprised to observe that this inhibition can be sequence dependent (*30,59,67*). In particular, base sequences which include a G quartet (i.e., four guanine bases in succession) have been shown to inhibit gene expression in a manner specific to this sequence (*68*).

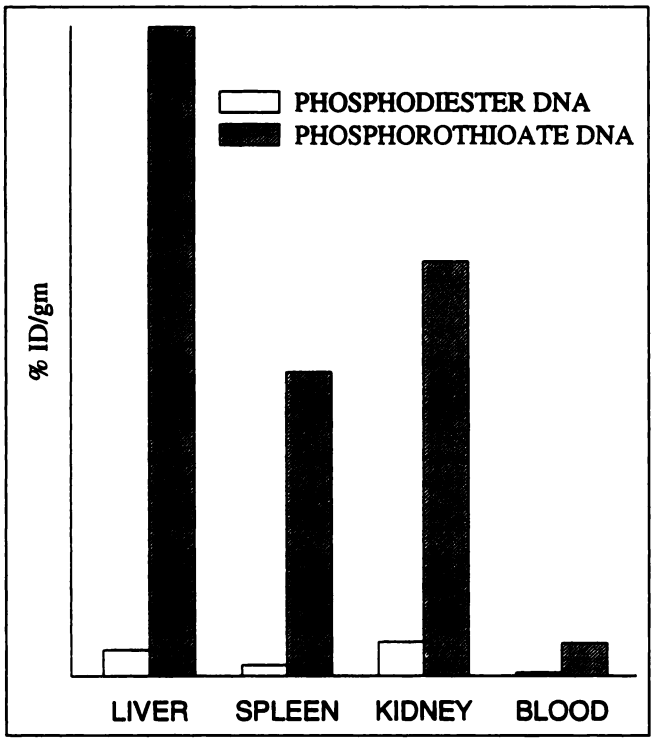

FIGURE 4. Histograms show accumulation (in %ID/g) of ^{99m}Tc in major organs and blood, 4 h post-intravenous administration to normal mice of single-strand phosphordiester DNA (white bars) and phosphorothioate DNA (shaded bars).

In a clear example of the effect of protein binding on the use of phosphorothioate DNAs for imaging, slow whole body clearance and accumulation of ^{99m}Tc radiolabel in the liver and other organs was observed in mice at levels interfering with imaging (*69*). The histograms in Figure 4 show the accumulation of radioactivity in major organs and blood 4 h post-intravenous administration to normal mice of 3–4 μg of a 22-base single-strand phosphodiester and uniformly modified phosphorothioate DNAs, both radiolabeled identically with ^{99m}Tc, using SHNH (Hynic) as a chelator. Whereas radioactivity levels are relatively low with the phosphodiester DNA (due, at least in part, to nuclease degradation), levels after adminstration of the phosphorothioate DNA are much higher because of protein binding.

While the influence of protein binding on antisense chemotherapy can be handled by increasing to saturation levels the dosage administered, it is doubtful whether the interference of protein binding can be handled so easily in antisense imaging. At a minimum, saturation of the protein sites would probably require a large administration of (probably nonspecific) phosphorothioate DNAs before the administration of the radiolabeled antisense DNA. It is hoped that second-generation DNAs will show lower protein binding affinities by eliminating the phosphorothioate groups.

Pharmacokinetics

Pharmacokinetic requirements differ sharply between antisense chemotherapy and imaging. Because cellular transport is limited and because mRNAs are produced continuously, chemotherapists prefer prolonged blood half-lives, even to the extent of developing timed-release approaches. DNAs with slow plasma clearance would help eliminate the need for multiple administrations. By contrast, antisense imaging requires rapid plasma clearance and rapidly improving target/nontarget radioactivity ratios for early imaging. Recent chemotherapy studies are focusing on oral administration (*70*), which would probably be of little or no value to nuclear medicine.

Antisense imaging is distinguished from antisense chemotherapy by a concern for the fate of the radiolabel rather than the fate of the carrier antisense DNA or the efficacy of chemotherapy. Sufficient evidence exists that antisense DNAs do accumulate in cells and, if properly labeled, their radiolabel will accumulate in cells as well. However, antisense chemotherapeutic drugs probably behave in vivo similarly to other chemotherapeutic drugs, such as, for example, cisplatin. These drugs show specific effects on, but not specific localization in, target tissues. For this reason, chemotherapeutic agents are seldom considered potential radiopharmaceuticals. No plausible mechanism exists to suggest that antisense DNAs are preferentially transported into cells in an antisense-specific manner. Therefore, adequate target-to-nontarget radioactivity ratios for imaging will be achieved only if the clearance of the radiolabel is accelerated in nontarget tissues versus target tissues. Preferential accumulation of the radiolabel in target tissues would then be the direct result of preferential retention of the radiolabel in the target, presumably by antisense mechanisms. There is some evidence that this may be the case for radiolabeled antisense phosphorothioate DNAs (*71,72*). The same evidence also suggests that the kinetics of hybridization and clearance are sufficiently rapid to permit the use of short-lived radionuclides, such as ^{99m}Tc and ^{111}In.

One clear limitation to phosphorothioate DNAs is their inability to cross the intact blood brain barrier (*10*). There is at present no satisfactory (i.e., short of intracranial adminstration) method of antisense targeting in this organ.

Toxicity

Among the first generation of antisense DNAs, only the phosphorothioates have been studied extensively in animals and in humans. The toxicities of the phosphorothioate DNAs have been attributed to the protein-binding affinities of this DNA and its negative charge. In monkeys, the principal dosage-limiting toxicities are hypotension and bradycardia, probably associated with complement activation, and prolonged clotting time, possibly associated with thrombin inactivation (*70,73*). In addition to transient toxicities, very high dosages of phosphorothioate DNAs can also cause changes in renal morphology. However, appreciable toxicity in animals was observed only after prolonged administration of dosages greater than 2–20 mg/kg (*22,73*). In patients, antisense DNAs have been administered intravitreally, intradermally and intravenously, the latter at dosages up to 2

mg/kg, without significant toxicity (*22*). Phosphorothioate DNAs have also been found to be nonmutagenic (*74,75*).

Chimeric DNAs have reduced abilities to activate complement over the phosphorothioate DNAs, presumably because of lower protein-binding affinities (*76*). They may be expected, therefore, to show less toxicity. However, these second-generation DNAs are still under investigation in animals.

One possible mode of toxicity inherent to antisense chemotherapy could result in collateral mRNA damage. Any antisense DNA may have the potential to bind, possibly with low affinity, to many mRNAs in addition to the target mRNA, as the result of unintended hybridization (*61*). This binding could interfere with the expression of normal genes and, consequently, with proper cellular function. There is at present no evidence for collateral damage.

Another possible mode of toxicity inherent to radiolabeled antisense DNAs may be more serious. In addition to emitting penetrating gamma rays, most radionuclides used for imaging in nuclear medicine emit Auger electrons to varying degrees. For example, ^{111}In, a potential radiolabel for antisense DNA imaging, has been considered a therapeutic as well as a diagnostic radionuclide, because of its decay by several Auger and conversion electrons (*77*). The high linear-energy transfer (LET) of these low-energy electrons poses a potential radiation hazard to biomolecules within a small volume surrounding the decay. In the case of antisense DNAs migrating into the cellular nucleus, the potential for chromosomal radiation damage, especially irreparable double-strand DNA breaks, is a concern. There is considerable evidence that Auger-emitters ^{125}I and ^{77}Br, when incorporated into the genomic DNA, are as efficient in producing cell killing as are densely ionizing α particles (*78*). In the case of ^{111}In, it was not necessary for the radioactivity to be deposited within the nucleus to produce radiation effects. After Chinese hampster cells in culture were uniformly labeled using ^{111}In-oxime, both chromosome and chromatid breaks were detected (*79*). These aberrations were shown to be the result of the radiation and not the oxime. Radiation effects depend on the radiation dose and dose rate, as well as the radiation sensitivity of the cell type, but, overall, the results of these and other investigations imply that radioactivity localizing by antisense mechanisms should be considered potentially as hazardous as that localizing by certain other nuclear medicine imaging procedures. Fortunately, the radiation toxicity of antisense DNAs radiolabeled with ^{99m}Tc instead of ^{111}In will be less of a concern, because of fewer emissions of Auger and conversion electrons in the decay of ^{99m}Tc (*80*). Of course, radiation toxicity will be an advantage for antisense radiation therapy (*21*).

In summary, antisense DNAs are much less toxic than conventional chemotherapeutic drugs. In addition, DNAs will be administered at low dosages for antisense imaging. For example, specific antisense imaging in pigs has been reported after dosages of only 10 μg (*19*).

Targets

Should antisense imaging become a reality, the majority of targets for antisense chemotherapy now in preclinical and clinical trials would immediately become candidates for imaging. For example, protooncogenes, such as c-myc, are overexpressed on multiple leukemias and solid tumors and should serve as potential targets for broad-spectrum tumor imaging agents (*81*). Likewise, antisense imaging in viral infections, such as HIV, could be useful in determining the location and extent of disease. The imaging of inflammatory processes or bacterial infections would also be of obvious value. In these cases, antisense imaging might be accomplished by simply radiolabeling and administering the therapeutic antisense DNA.

Obviously, it will also be useful to target diseases that may be of limited interest for chemotherapy, such as ischemic heart disease and renal insufficiency (*71*). Moreover, it may be of interest to image the expression of mRNAs, which may be involved in normal cellular function and therefore ineligible for treatment. As one example, heat shock proteins are synthesized in response to a variety of cellular stresses, including hypoxia (*82*). The concentration of mRNA coding for one of these proteins (HSP70) has been shown to be elevated in ischemic myocardial tissue (*83*). Possibly a radiolabeled antisense DNA against this mRNA might provide useful images in myocardial ischemia and infarction. However, the difficulties and expense of developing imaging agents in this way are considerable. It should first be established that the mRNA to be targeted is present in sufficient concentration to allow imaging (i.e., that the gene in question is sufficiently expressed). Then, assuming that the sequence of the targeted mRNA is available, a large number of antisense DNA sequences must be tested in tissue culture to identify those showing the most effective binding and retention (*3*). Recent developments in combinatorial approaches, using immobilized arrays of random-sequence DNAs exposed to radiolabeled mRNA, may simplify this process (*84*). However, these investigations should be performed ideally with the DNAs radiolabeled, if the label has any likelihood of influencing critical properties. Finally, dosage-response studies using proper sense, scrambled, mismatched and nonsense DNA controls, with measurements of mRNA and protein concentrations in various cell types and tissues, should be performed to confirm that localization is antisense and selective (*59*). The possiblility of genotoxicities should also be investigated. Many academic laboratories will not have the necessary resources for these studies.

Radiolabeling

Methods for radiolabeling single-strand and double-strand DNAs with β-emitters such as ^{32}P, ^{35}S, etc. and with gamma-emitting radioisotopes of iodine have been in use for years (*85*). Recently, radioiodination methods for DNAs have been brought up to date (*86,87*).

Most radioisotopes used in nuclear medicine are metals. One straightforward approach to radiolabeling DNAs with

metallic radionuclides is first to derivatize the antisense DNA on either of its ends with a primary amine, possibly attached by a suitable linker to minimize steric hindrance. The amine then may be conjugated with various metal bifunctional chelators, such as the anhydrides of diethylenetriamine pentaacetic acid (DTPA) or the N-hydroxysuccinimide esters of SHNH and mercaptoacetyltriglycine (MAG3). Single-strand DNAs have been radiolabeled in this manner with ^{67}Ga, ^{111}In and ^{153}Sm (*19*). Antisense DNAs can display rapid pharmacokinetics, and, if the kinetics of cell membrane transport are also rapid, the radionuclide of choice for antisense imaging will often be ^{99m}Tc. Single-strand DNAs have also been radiolabeled with this radionuclide (*69,88,89*).

Thus it appears that conventional methods used to radiolabel antibodies and peptides with ^{99m}Tc may be used without modification to radiolabel DNAs. Radiolabeling DNA might even be simpler, since DNAs can be exposed safely to temperatures and chemical environments during labeling that would denature many proteins and peptides. Occasionally, DNAs radiolabeled with metallic radionuclides have been tested to ensure that, after labeling, they can still hybridize to their complement (*69,90*). Unfortunately, the effects of radiolabeling DNAs on DNA cell membrane transport, on targeting mRNA and on clearance of the label from nontarget tissues, have not been evaluated.

One additional consideration relevant to radiolabeling in antisense imaging concerns the specific activity of the labeled DNA, since this parameter affects tumor counting rate. Assuming a reasonable value of 10,000 target mRNA/cell (*91,92*) and 10^9 cells/g of tumor, it can be calculated that 10 μCi/gm may accumulate in the tumor as the result of antisense binding, if the DNA is radiolabeled at a specific activity of 100 μCi/ug. Since this specific activity is easily achievable (*69,90*), tumor counting rates should not be limited by low specific activity. In general, encouraging results obtained in one study with ^{111}In-labeled DNA suggest that radiolabeling of antisense DNAs with imageable (or therapeutic) radionuclides is unlikely to pose major problems (*72*).

Since single- and double-strand DNAs are routinely radiolabeled at high specific activities with ^{32}P and other β emitters without interfering with hybridization (*93*), there is little likelihood that radiolabeling, at least for antisense imaging, will interfere with hybridization through radiation damage to the nitrogenous bases or other DNA structures.

The foregoing was an overview of the issues surrounding the development of antisense imaging. Obviously, many difficulties remain. Fortunately, several studies related to antisense imaging have been reported with generally positive results. These studies offer some optimism that antisense imaging may eventually become a reality.

PIONEER ANTISENSE IMAGING INVESTIGATIONS

A small number of investigations related to the use of antisense DNAs for imaging have been reported (*71,72,94,95*).

A large (67-base) phosphodiester single-strand antisense DNA designed to target the mRNA encoding serum albumin has been administered to normal rats (*94*). The DNA was conjugated covalently with polylysine to neutralize the negative charge and with asialoglycoprotein for specific delivery to the liver. These modifications may have improved the in vivo stability of this phosphodiester DNA. Using ^{32}P as a label, rapid (within 1 h) accumulation into the liver was observed. Some evidence was presented that the DNA ultimately targeted the nucleus. However, no sense or other control DNA was used to show that localization was achieved by an antisense mechanism. Imaging would have been unfavorable, since up to half the administered radioactivity at all time points remained in tissues outside the liver.

One investigation demonstrated in vitro what appears to be preferential antisense intracellular retention (*71*). An 18-base uniform phosphorothioate single-strand DNA was synthesized antisense to the initiation codon of the erbB2 oncogene mRNA expressed in the MCF7 mouse mammary cell line. The sense strand was also synthesized and both were radiolabeled with ^{32}P. The uptake and retention in tissue culture of the labeled antisense and sense DNAs were determined in the MCF7 cell line and in a nonspecific cell line (MOPC315). After the first hour of incubation, greater accumulation of the antisense DNA occurred in the MCF7 cell line, and, thereafter, the antisense DNA was preferentially retained. However, accumulation and retention by a sequence-specific, but nonantisense, mechanism is always a possibility.

The most ambitious study reported thus far, and one of only two reported attempts at imaging, is that of Dewanjee et al. (*72*). These authors synthesized both a phosphodiester and a uniform phosphorothioate single-strand DNA, each 15 bases long with an unspecified sequence, complementary to a sequence within the initiation codon site of the c-myc oncogene mRNA. Both the antisense and sense strands were prepared. All four DNAs were conjugated with benzyl-isothiocyanate DTPA via a six-carbon methylene spacer to the amine-derivatized DNAs, and all were radiolabeled with ^{111}In. Possibly because of the labeling method, the phosphorothioate DNAs did not show the expected increased serum or tissue protein binding over the phosphodiester DNAs, either by high-performance liquid chromatography analysis of mouse plasma incubates or in the results of mouse biodistribution measurements. In an apparent demonstration of antisense localization, uptake of the radiolabel in murine monocyte leukemia tumor cells in tissue culture appeared to be significantly higher for both antisense DNAs over both sense DNAs. The authors also showed that specific binding to isolated mRNAs was about 20-fold higher for the labeled antisense compared to the sense DNAs. The most encouraging results were obtained in nude mice bearing a mammary adenocarcinoma, in which 10%–12% of the radioactivity on both injected antisense DNAs and only 1% on the sense DNAs localized in tumor at 1 h postadministration. Figure 5 is taken from this work (*91*) and shows the increased uptake

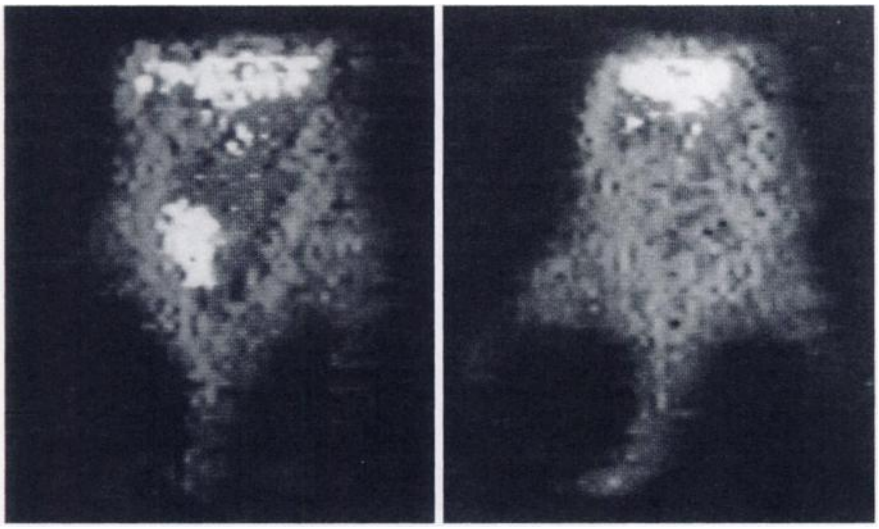

FIGURE 5. Whole-body images of mice bearing abdominal mammary tumors 2 h post-intravenous administration of antisense (left) and sense (right) DNAs labeled with ^{111}In. Increased uptake of radiolabel in tumor is readily apparent in antisense image [Reprinted with permission of (*91*)].

of the radiolabel in the xenograft at 2 h after antisense administration (left panel) compared to sense administration (right panel). Among the many encouraging results of this investigation is the observation that, when radiolabeled by one method, first generation DNAs accumulate preferentially in tumor tissue and do so rapidly, so that successful imaging may be accomplished in 1–2 h post-intravenous administration.

In a recent study, a 23-base phosphodiester DNA was radiolabeled with ^{125}I via a tyramine group conjugated to the 5′ end (*95*). The DNA was complementary to the initiation codon site of the mRNA coding for TGF α, a growth factor associated with tumorigenesis. After intratumoral administration in nude mice with mammary tumors expressing high levels of the TGF α mRNA, the biodistribution of the radiolabel was determined over time. Approximately 80% of the radiolabel was transferred within 1 h from the tumor to the abdomen, although sufficient activity (1%) remained in the tumor to provide positive images 24 h postinjection. In vitro studies in cell culture showed unmistakable evidence of cell membrane transport of the radiolabel and predominant accumulation in the nucleus within 1 h of incubation. Extraction of DNA from the cell pellet and analysis by gel electrophoresis showed no evidence of in vitro instability of the DNA during 8 h postincubation. This surprising stability in vitro for a phosphodiester DNA is probably related to the tyramine group on the 5′ end, which should have restricted digestion by 5′ exonucleases. That there was no long-term retention of radioactivity in any tissue except tumor and thyroid (the latter the result of dehalogenation) is suggestive of digestion in vivo. No control DNA was used in this investigation, therefore it is not possible to attribute retention of the radiolabel in tumor to an antisense mechanism.

DISCUSSION AND CONCLUSION

In the clinic, antisense chemotherapy is less than a decade old. The field is not yet established, and some fatal flaw in the approach may still emerge. More likely, given the encouraging preliminary results in ongoing trials and the considerable resources devoted to improving therapy, superior chemotherapies using antisense will materialize. These novel chemotherapies may bear little resemblance to those now in use. For one example, small molecules have been shown to bind mRNAs and double-strand DNAs in a sequence-dependent manner and with affinities comparable to that of antisense DNAs (*96*). Perhaps small molecules with superior in vivo properties of stability and pharmacokinetics and with improved cell membrane transport may eventually replace DNAs as antisense drugs.

The purpose of this article was to provide a brief description of antisense chemotherapy and to address the question of whether antisense localization can be applied to nuclear medicine imaging. Clearly, antisense imaging would be an extremely valuable diagnostic tool, since, in theory, almost any tissue or disease state could be selectively imaged. As this contribution may make clear, however, many improvements in the current state of antisense localization will be needed to reach this nirvana.

Consider first the chemical form of DNA: phosphorothioate DNAs are not ideal for antisense imaging. Possibly methylphosphonate DNAs or, more likely, the second-generation of chimeric DNAs will show superior pharmacokinetics and cell membrane transport. Conceivably, a chemical form of DNA found unsuitable for antisense chemotherapy might be the DNA of choice for antisense imaging. This would be the case if that DNA provided prolonged retention of the radiolabel in target tissues and rapid clearance from nontarget tissues. Consider the method of radiolabeling: favorable properties of radiolabel retention and clearance depend not only on the chemical form of DNA but also on the method of radiolabeling. Existing methods of radiolabeling DNAs with gamma emitters may possibly decrease cell membrane transport, interfere with mRNA binding or show intracellular instabilities leading to prolonged nonspecific retention. Alternative methods of radiolabeling will then be needed. Finally, consider the mRNA target: for good rea-

sons, early antisense imaging will probably focus on those targets already established for antisense chemotherapy. Eventually, there will be interest in targeting mRNAs related to other disease states of specific interest in nuclear medicine. To do so will require extensive in vitro studies to identify the proper antisense DNA sequences for imaging.

The task of developing antisense imaging appears formidable. Fortunately, some remarkably positive results have already been obtained in tissue culture and, especially, in animal studies. Studies in tissue culture have shown that phosphorothioate DNAs radiolabeled with ^{32}P can accumulate preferentially and rapidly in tumor cells expressing the mRNA target, at least under certain circumstances (*71*). Studies in tumored mice also showed preferential accumulation and respectable tumor/normal tissue ratios of ^{111}In radiolabeled phosphorothioate and phosphodiester antisense DNAs within 1–2 h postadministration (*72*). The results of these studies encourage the belief that phosphorothioate DNAs may be suitable (if not ideal), that existing radiolabeling methods do not interfere with antisense imaging and that adequate target/nontarget ratios can be achieved rapidly. If studies such as these can be confirmed, especially in a variety of other tumor types as well as nonmalignant tissues, this will prove that antisense imaging can be accomplished adequately with existing technologies. Nevertheless, antisense imaging as performed today will almost certainly require improvement. The potential of antisense imaging and the encouraging results now being obtained with antisense chemotherapy suggest that the effort needed to exploit antisense localization for imaging and radiotherapy will be worthwhile.

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