Molecular Targeting with Radionuclides: State of the Science*

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Inherent in the application of advances in biomedical science to nuclear medicine is the concept of molecular targeting: the in vivo concentration of labeled tracer by a gene, its transcribed DNA, or its protein product. This mechanism of localization has been and is being exploited for both nuclear imaging and radioisotopic therapy. Agents, such as antisense molecules, aptamers, antibodies, and antibody fragments, can be aimed at molecular targets. Tumor and nerve cell receptors provide such targets. So do certain cellular physiologic activities, including metabolism, hypoxia, proliferation, apoptosis, angiogenesis, response to infection, and multiple drug resistance. In this article we review the principles of molecular targeting based on radioisotopic methods and provide examples from the literature. We discuss applications to imaging and therapy and point out the hurdles that must be overcome in bringing molecular targeting to clinical reality.

Key Words: molecular targets; molecular imaging; radioisotopic therapy

J Nucl Med 2003; 44:1945–1961

The discovery of techniques for the manipulation of nucleic acids, including bacterial DNA restriction systems, DNA sequencing, reverse transcription, and polymerase chain reaction, has catalyzed an explosion of knowledge of the molecular basis of human disease, with >93,000 molecular biology-related Medline citations appearing during the year 2001 alone (authors' informal count). Equipped with this new understanding, 21st Century medicine is poised to implement a new class of therapeutics, based on detailed models of the processes of disease, with exquisite specificity worthy of Ehrlich's notion of the "magic bullet." While every branch of medicine is likely to undergo eventual transformation by this revolution, nuclear medicine has already begun to feel its effects and is likely to be profoundly, if not unrecognizably, altered in the not-too-distant future.

WHAT IS MOLECULAR TARGETING?

"Molecular targeting" may be defined as the specific concentration of a diagnostic tracer or therapeutic agent by virtue of its interaction with a molecular species that is distinctly present or absent in a disease state. The molecular species in question could be a mutated locus of DNA or its protein or RNA product; a gene product of normal sequence and structure, aberrantly expressed in a given tissue; or a transcriptionally normal gene product whose structure or function has been modified by abnormal RNA splicing or posttranslational processing. Molecular targeting agents differ from conventional physiologic tracers, such as radioiodide or myocardial and cerebral perfusion indicators, in that physiologic tracers are directed toward processes characteristic of a normal tissue or cell type and are dependent on the cooperation of multiple gene products or on nonspecific bulk processes, such as diffusion, membrane permeability, or electrostatic interactions.

Implicit in the molecular paradigm of disease description is the assumption that diseased or injured cells are different from their normal neighbors—specifically, that they demonstrate a distinctive "pathotype" or signature pattern of altered expression or processing of gene products. This pattern would be common to cells of similar origin if subjected to the same disease process. Thus, even cells subjected to passive injury, such as trauma, ischemia, or bacterial invasion, will respond by upregulating response elements, such as heat-shock proteins, interferons, or hypoxia-inducible factor, or, when adaptation fails, by activating caspases and other apoptotic factors during the final throes of cell death. Characterization of such pathotypes up till now has relied on painstaking hit-or-miss methods of analyzing one gene product at a time but recently has been accelerated by the introduction of genomic and proteomic methods of mass screening.

As the most sensitive noninvasive modality presently available for the detection and mapping of pathotypic markers, nuclear medicine enjoys a strategic position at the forefront of this trend. As will be seen below, many of these

Received Apr. 7, 2003; revision accepted Sep. 25, 2003.

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markers or their natural ligands can be more or less directly translated into potential imaging agents. These agents can be used to establish a primary diagnosis; to guide therapy by demonstrating the presence of appropriate molecular targets; to confirm delivery of gene therapy agents; to assess response after therapy; and to evaluate new therapeutic compounds. Many agents, labeled with cytocidal β- or α-emitting radionuclides instead of γ-emitters, will also be suitable for targeted radiotherapy.

In this article, we will review the possibilities for molecular targeting and include discussions of how molecular imaging and targeted radionuclide therapy work, what clinical applications are now available, what new agents are under development, what frontiers remain, and, finally, what fundamental limits to molecular targeting are likely to be encountered. The focus will be primarily on radioisotopic methods, as opposed to MRI agents or optical-fluorescence approaches, since these are likely to have the most general clinical application in the near term. Reviews of alternative methods are presented elsewhere (*1–3*).

HOW DOES MOLECULAR IMAGING WORK?

As with other imaging applications, molecular imaging requires fixation of a signal-emitting molecule (e.g., one containing a radioactive, fluorescent, or paramagnetic label) within the cell or tissue where the target is expressed. There are several different mechanisms by which this can be brought about. In the receptor–ligand model, one signal molecule (the ligand) binds with high affinity to a specific site on a target molecule (the receptor), which has a stationary biodistribution over the period of imaging. If multiple binding sites are present, a single receptor can bind more than one ligand; however, the maximum stoichiometry will always be a low multiple of the number of receptor molecules available. The key determinants of success for this type of imaging are the specificity and affinity of the receptor–ligand interaction, as well as the receptor density itself. The receptor–ligand pair does not necessarily have to mimic a naturally occurring interaction. For example, peptides derived artificially from phage display can bind their targets with affinities equal to those of endogenous ligands for their receptors and, indeed, can be made to bind with high specificity to molecules that are not considered to have any physiologic receptor activity at all.

The antigen–antibody model is essentially a variant of the receptor–ligand model, although it follows a somewhat more complex stoichiometry and binding kinetics. Again, the critical parameters are specificity, affinity, and the density of antigenic epitopes. However, since the antibodies in use are typically divalent, the possibility of cross-linking can lead to an interaction that is stronger than would be predicted from the direct binding affinity. This effective strength of interaction, termed avidity, can be empirically determined but bears a complex relationship to the binding affinity and to the density and geometry of available epitopes.

In the transporter–substrate mode, the signal molecule is concentrated within a cell or tissue compartment via a mechanism in which the activity of the target molecule is the rate-limiting factor. Although the signal molecule may be trapped in the compartment by subsequent modification, this modification is not rate-limiting, and the transporter itself does not chemically alter the substrate. The transporter may have multiple substrates (e.g., both glucose and other hexoses); however, the signal molecule must have a restricted interaction with a single transporter. Since many transporters have multiple isoforms, this can be effectively dependent on context. Furthermore, the transporter must be uniquely present or absent in a process of disease. Thus, FDG can be considered a molecular targeting agent for Glut-1 transporters in bronchogenic carcinoma or in ischemic myocardium, because Glut-1 is not expressed at equivalent levels in healthy lung or heart. In the context of hepatobiliary imaging, however, diisopropyl iminodiacetic acid would not be considered a molecular targeting agent because the anionic-binding carrier protein that is responsible for its uptake into hepatocytes is expressed both in healthy people and in patients with cholecystitis, and diagnosis is based on the pattern of bulk flow of excreted tracer.

In the enzyme–substrate model, the target molecule is an enzyme that chemically modifies the signal molecule, fixing its biodistribution. Unlike receptor–ligand binding, there is no fixed stoichiometry. A single molecule of enzyme can interact with many substrate molecules, multiplying the signal manyfold. A very rare target can be detected, provided that it has a high turnover (k_{cat}) and a high affinity (low K_m) for the signal molecule as a substrate. A key issue in enzyme targeting is physical delivery of the substrate. Some enzymes, such as matrix metalloproteinases, are extracellular in location. Many desirable targets, however, are restricted to specific intracellular compartments, where access to substrates is tightly controlled. This is quite different from the case of receptors, many of which are accessible at the cell membrane. Thus, an effective target molecule either must be capable of rapid diffusion into the cell or must use a transport mechanism with high throughput characteristics.

In a complex or hybrid model, the signal molecule does not react directly with the target. Instead, targeting is a 2-step process, using an intermediary that first binds the target directly. In the second step, the signal molecule reacts with the intermediary, which fixes its biodistribution. As an example, the intermediary might be a biotinylated antibody, and the signal molecule might be a streptavidin-linked chelator labeled with ^{99m}Tc. This approach can improve the specificity of targeting with agents that have a high affinity but clear slowly from the blood pool or have slow penetration into tumors. It can also improve the sensitivity of receptor targeting, if the intermediary binds the receptor first and then amplifies the signal by catalytically acting on the signal molecule as a substrate.

HOW DOES TARGETED RADIOTHERAPY WORK?

In 1926, the eminent radiobiologists Regaud and Lacassagne predicted that "the ideal agent for cancer therapy would consist of heavy elements capable of emitting radiations of molecular dimensions, which could be administered to the organism and selectively fixed in the protoplasm of cells one seeks to destroy" (*4*). This prescient statement is still a workable definition of targeted molecular radionuclide therapy. By heavy elements, the two French radiobiologists were probably referring to α-particle–emitting nuclides but they might well have included other emitters whose particulate radiations extend no further than molecular or cellular dimensions, thus sparing neighboring cells and normal tissues.

We are now aware that radiation that damages DNA stimulates cells to take several courses of action (*5*). Cells may be held at checkpoints in the cell cycle, $G_1 \rightarrow S$ or $G_2 \rightarrow M$, while repairs are contemplated or made, or a decision is made to have the cells enter programmed cell death or apoptosis. Apoptosis, a mechanism of cell death that incurs little or no inflammatory response, is used in embryologic modeling as well as in response to noxious agents that jeopardize the future of the cell. The pathways leading to apoptosis involve a complex of signal transduction proteins; in the case of radiation response, the gene product p53 plays a central role. Lymphoid cells are more susceptible to apoptosis in the first instance, and it may be that the favorable response of certain lymphoid tumors to radioimmunotherapy owes its success to this circumstance. Other tumor cells, especially those of epithelial origin, generally die of reproductive failure after one or more rounds of division, although these too may be held at cell-cycle checkpoints. Their ultimate fate is yet to be elaborated, but the endpoint of tumor sterilization has the same result as immediate cell death. Effectiveness of radiation in producing reproductive cell death is dependent on dose rate as well as ionization density. It is for the latter reason that α-particle and Auger electron emitters are being closely examined as labels for targeted molecular radionuclide therapy.

Recently, it has been shown that tumor cells surrounding those killed directly by densely ionizing radiation may also suffer reproductive failure (*6*). If this occurs clinically, it may be a means of amplifying the effect of such therapeutic agents and an obviation of the requirement that all cells in a tumor be labeled.

WHAT HAS BEEN ACCOMPLISHED SO FAR?

Imaging Applications

Several molecular imaging agents have already found their way to clinical use. Foremost among these is ^{18}F-FDG, which in certain contexts (i.e., where hexokinase activity is not rate-limiting and where intracellular phosphatase activity is constitutively low) can be treated as a marker of glucose transporter expression. Touted as the "molecule of the millennium," ^{18}F-FDG has virtually single-handedly primed the recent prodigious expansion of PET as a routine clinical imaging tool. It has been shown to be a sensitive agent for tumor detection, with uptake that correlates well with the grade or biologic aggressiveness of several tumor types. It can also play a role in monitoring response to therapy. Established nononcologic applications include assessment of myocardial viability and of interictal cerebral hypometabolism in epilepsy. An important limitation of ^{18}F-FDG imaging is its nonspecificity, since the tracer can be taken up by inflammatory foci, and enhanced tumoral uptake can often be seen as an early response to therapy. Two somatostatin receptor–targeting agents, ^{111}In-octreotide and ^{99m}Tc-depreotide, have found use in tumor imaging, the first for neuroendocrine tumors, such as carcinoid, and the second for carcinoma of the lung. ^{131}I- and ^{123}I-labeled metaiodobenzylguanidine (MIBG), a marker for the adrenal norepinephrine reuptake transporter, plays a role in the detection of pheochromocytoma and neuroblastoma. Although antibody imaging has yet to achieve its full potential, several antitumor antibodies have shown proven usefulness in certain clinical settings. These include ^{111}In-satumomab pendetide (Oncoscint), targeting the mucin-like surface glycoprotein TAG-72; ^{111}In-capromab pendetide (Prostascint), targeting the prostate-specific membrane antigen; and ^{99m}Tc-arcitumomab (CEAScan), targeting cell-membrane–bound carcinoembryonic antigen (CEA). ^{99m}Tc-Apcitide (AcuTect), a peptide agent that targets the glycoprotein IIb/IIIa receptor on activated platelets, is used for detecting deep venous thrombosis. These examples clearly demonstrate that the era of molecular imaging lies not beyond the horizon but is already at hand.

Targeted Radiotherapy Applications

The most successful targeted radionuclide therapy procedures have aimed at receptors located on the surface of cancer cells. The simplest are somatostatin analogs labeled with several radionuclides (*7*). These have included a hard β-emitter, ^{90}Y, whose particles have a range of several millimeters (the coordination chemistry of ^{90}Y is similar to that of ^{111}In—hence, the easy substitution of the therapeutic radionuclide for the diagnostic one), an Auger cascade emitter, and a soft β-emitter, ^{177}Lu (*8*). Early clinical results seem to favor the ^{177}Lu label, but it is unclear whether this is due to the radionuclide or the enhanced binding capacity of the modified octreotide used.

More effort has gone into antitumor antibodies and their fragments than other agents and there is ever-growing literature on their application (*9–11*). The most successful results have been with lymphomas, particularly B-cell lymphoma, but the mechanisms are somewhat unclear. Even unlabeled antibodies, such as rituximab, are cytotoxic, and the radioactive emission in these cases exerts an adjunctive effect. Part may be due to the high radiosensitivity of these tumors and their death by apoptosis; part may be due to the accessibility provided to the targeting agents compared with

solid tumors whose architecture precludes easy entry and uniform distribution of large immunoglobulin molecules.

^{131}I-MIBG has been used for the palliative therapy of metastatic neuroblastomas (*12*). Preliminary uptake studies with ^{123}I or ^{131}I tracer quantities can be some guide to response.

WHAT IS IN THE PIPELINE?

Antisense Targeting

Antisense imaging strategies exploit the exquisite specificity of nucleic acid base-pair binding. The frequency of occurrence of a specific RNA or DNA nucleotide sequence is $1/4^n$, where n is the number of bases in the sequence. Thus, a 15-mer sequence would be expected to hybridize randomly only once, at most, in the 3×10^9 base pairs of the human haploid genomic DNA complement. The affinity of base-pair binding is also quite high, with an 11-mer sequence binding with picomolar affinities, although a single base-pair mismatch in the middle of a short oligonucleotide sequence can decrease the melting temperature of an RNA:DNA hybrid by up to 14°C. Oligonucleotides in the range of 20–30 base pairs can be easily synthesized in quantity by automated processors, so in theory any gene or RNA transcript could be targeted.

Unlabeled antisense agents have been introduced in several therapeutic trials. These are believed to inhibit gene expression at 2 levels. The first, transcriptional arrest, requires entry into the nucleus, access to chromatin-bound DNA sequences, and possibly triple-helix formation; alternative mechanisms may involve interference with intron splicing, capping, and polyadenylation. At the second level, translational arrest, antisense DNA acts as a stimulant for ribonuclease H-mediated degradation of messenger RNA (mRNA); however, there may also be an effect at the ribosomal level to inhibit translation directly (*13*). Given the complexity of these interactions, a mechanistic model for therapeutic antisense agents must necessarily be complex. However, when used for imaging applications, an ideal antisense agent would function along the lines of the receptor–ligand model described above.

The principal technical problems for all antisense applications consist of directing the delivery of the nucleic acid agent to its target within the cell and maintaining the stability of the agent while it is en route. There are no direct receptors in the cell membrane for oligonucleotides. The uptake mechanism is nonspecific and relies on binding to cell-surface proteins or receptor-mediated adsorptive endocytosis or pinocytosis (*14–16*). Consequently, several experimental studies have relied on mass effect, injecting large quantities of naked DNA to ensure uptake. However, the efficiency of uptake can potentially be improved by using liposomes or viral vectors for delivery or by neutralizing the strong negative change of the DNA by complexing it with polylysine. No antisense vehicle has yet been developed, however, which can cross the blood–brain barrier.

Both extracellularly and intracellularly, oligonucleotides are subject to action by exonucleases and endonucleases, which rapidly hydrolyze an unmodified phosphodiester DNA or RNA probe to single nucleotides. However, stability can be much improved by using modified DNA, such as methylphosphonates and phosphorothioates. Yet another approach is to use chimeric oligonucleotides, which contain DNA with gaps made of 2′-modified RNA.

Since uptake of the probes is a bulk process, and specificity only appears at the point of hybridization with the target, any successful strategy for imaging or radiotherapy must also include a means for disposing of probes that do not hybridize successfully. This factor significantly limits the signal-to-background ratio and has been the Achilles' heel of many imaging approaches to date.

In addition to canonical DNA:DNA and DNA:RNA hybridization, oligonucleotides can also bind to intracytoplasmic and intranuclear proteins, both in a sequence-specific and nonspecific manner. Unfortunately, many published imaging studies have failed to show that antisense oligonucleotides actually bind to their target mRNA sequences.

Unlabeled phosphodiester and phosphorothioate oligonucleotides have not been shown to be mutagenic. However, caution must be exercised in applying radioactive labels to agents that localize in the nucleus, including those for detection of mutations or chromosomal rearrangements, since the emission of Auger electrons or low-energy β-particles in the immediate vicinity of nuclear DNA would invariably damage chromosomal DNA.

Antisense oligonucleotides have been tested in several imaging applications, including viral infections (HIV, cytomegalovirus, Epstein–Barr virus), inflammation, and myocardial ischemia (heat shock protein 70) (*17*). Recently, a 15-mer antisense phosphorothioate DNA complementary to c-*myc* oncogene mRNA, labeled with ^{111}In-benzylisothiocyanate diethylenetriaminepentaacetic acid (DTPA), was shown to have specific uptake in cultured murine monocyte leukemia tumor cells (*18*) and mouse mammary adenocarcinoma (*19*). A 23-mer phosphodiester DNA oligonucleotide complementary to transforming growth factor-α (TGF-α) mRNA with ^{125}I-tyramine showed increased uptake in murine mammary adenocarcinomas expressing high levels of this gene (*20*). Applications of this type are likely to become abundant in the near future.

Mutation Detection

In general, neoplasms arise via an accumulation of multiple somatic mutations within a clonal subpopulation of cells. In a given context, the distribution of these mutations is not entirely random. In some tumor types, stereotypic mutations or translocations are seen, which involve specific chromosomal sites or functional domains. Mutations in other genes, such as p53, are so common among different tumor types as to constitute a virtual marker for neoplasia. The ability to detect these mutations noninvasively would, in the appropriate setting, provide a means for early detec-

tion of cancer, for histologic characterization, for the prediction of treatment sensitivity, and for detecting the emergence of biologically significant transformations (e.g., of premalignant to malignant or of low-grade to high-grade transitions).

To date, little specific progress has been made in this area, although antisense technology may potentially serve as a foundation. A primary challenge to be overcome is the very low number of targets per cell. Some of the mutations in question are present in abnormally amplified chromosomal DNA or satellite chromosomes, offering hundreds or even thousands of targets per cell. Other mutations may affect transcribed regions of DNA, permitting indirect detection via mRNA copies. However, to be useful as a general method, mutation detection will ultimately require a mechanism for signal amplification, once the target has been bound.

Aptamers

Single-stranded oligonucleotides have a complex 3-dimensional structure and can interact with suitably conformed proteins with an affinity and specificity comparable to that of receptor–ligand binding. Systematic evolution of ligands by exponential enrichment (SELEX) (*21*) is an empiric technique for selecting oligonucleotides that will bind to a given target, using a repetitive process of selective binding and amplification by polymerase chain reaction. Typically, combinatorial libraries of 10^{14}–10^{15} sequences of up to a few hundred bases in length can be screened by this technique. Like phage display, SELEX offers the potential of rapidly designing radiolabeled ligands for almost any target. Nevertheless, there are some significant constraints to be considered. The relatively high molecular weight of successful aptamers (typically, 8–12 kDa) leads to poor membrane permeability and slow kinetics, both of uptake and of clearance of unbound aptamer from the cell (since no aptamer-specific transporters exist, uptake is necessarily nonspecific). Furthermore, single-stranded oligonucleotides are rapidly degraded by nucleases, both intracellular and blood-borne. As is the case for antisense oligonucleotides, chemically modified nucleotides, such as phosphorothioates and methylphosphonates, are likely to show improved stability; however, any modifications will have marked effects on the 3-dimensional conformation and binding affinity of the aptamer probe.

Tumor Antigens

Since the development of immunization technology for obtaining polyclonal rabbit and goat antibodies in the 1960s, followed in 1975 by the introduction by Kohler and Milstein of hybridoma technology for making monoclonal antibodies, specific markers have been developed for thousands of antigens associated with normal and neoplastic tissue. It is conceptually a simple step to attach a radioactive label to a well-characterized antibody to produce an in vivo imaging agent or, with β- or α-emitters, a therapeutic agent. However, experience with antibody imaging and therapy has met with several difficulties, and progress has been disappointingly slow.

Technical problems with antibody imaging include human antimurine antibody (HAMA) response, cross-reactivity, nonspecific uptake, hepatic uptake and metabolism with loss of labeling and immunoreactivity, and slow kinetics of uptake and blood clearance. The use of human or chimeric human–mouse antibodies has reduced HAMA response, and smaller F(ab′) or $F(ab')_2$ fragments show sufficiently rapid kinetics to permit ^{99m}Tc labeling in some cases. Delivery of antibodies to the target tissue can at times be impractical, however, as in the case of tumors with high interstitial fluid pressure or shielded by the blood–brain barrier. Furthermore, immunohistochemistry relies on physical and chemical methods for disrupting the cell membrane to gain access to intracytoplasmic and nuclear antigens. Such methods are not feasible in vivo, with the result that most of the successful antibodies have targeted cell-surface antigens or, as in the case of antimyosin antibodies, have relied on increased permeability of injured cell membranes to ensure delivery of antibodies to their targets.

Alterations in mRNA splicing or posttranslational modification of target antigens can also have a marked effect on antibody affinity and specificity or on elimination of circulating antibody–antigen complexes. Behr et al., for example, found profound differences in clearance rates of anti-CEA monoclonal antibodies in human patients with different tumors. These investigators speculated that different CEA-expressing tumor types produced heterogeneous CEA molecules and that the differences in antibody clearance were due to varying clearance rates of these circulating CEA species (*22*).

Nonetheless, substantial progress has been made. As noted above, diagnostic antibodies are now commercially available for imaging expression of CEA, tumor-associated glycoprotein (TAG-72) and prostate-specific membrane antigen. Anti-CD20 antibodies have recently been approved for therapy of low-grade or follicular lymphomas. Recently published work has targeted cytokeratin 8 (*23*), intercellular adhesion molecule-1 (ICAM-1) (*24*), polymorphic epithelial mucin antigen (MUC1) (*25*), and the epidermal growth factor receptor (*26*). Anti-CEA, already approved for imaging colon and ovarian cancer, has also been reported to show high specificity in imaging palpable and nonpalpable breast cancers (*27*).

Ongoing work also addresses improvements in the design of antibodies and in protocols for their use. Nishimura et al. have shown that recombinant λ light chain of the human monoclonal antibody HB4C5, which targets antigens found in many non–small cell lung carcinomas, was 40 times more immunoreactive than the parent antibody, and had superior uptake in the LC6 cell line and in nude mice with lung tumor xenografts (*28*). The λ light chain penetrated better into the tumor sites, especially in solid tumor areas.

Several recently reported protocols have attempted to improve specificity using multistep antibody targeting. With

this approach, an unlabeled bispecific antitumor–antihapten antibody is first injected, followed by a blocker to saturate the antihapten binding sites of the antibodies not bound to tumor. Imaging is then performed, by injecting a radiolabeled low-molecular-weight hapten, such as a ^{68}Ga chelate, which binds the tissue-bound antibody. Conjugation with polyethylene glycol has been shown to reduce immunogenicity and enhance circulating dose of antibodies (*29*).

Finally, an alternative approach is to eschew antibodies altogether and to use peptides with high binding affinities for the antigen of interest. Peptide sequences can be derived rationally, by inspection of endogenous binding sequences, by sequencing binding regions of successful antibodies, or, empirically, by phage display. In phage display, a combinatorial library of random nucleotide sequences (typically, 10–50 codons) is inserted into a gene encoding the coat protein of bacteriophage M13. The library is allowed to bind to the target of interest, and the most avidly binding phage are reisolated, pooled, and recultured. The process is repeated until a small set of phage with high binding affinity is isolated. The coat gene is then sequenced to determine the amino acid sequence of the peptide insert responsible for binding. The advantages of peptides include feasibility of direct synthesis, absence of interaction with F_c receptors, and faster uptake kinetics and blood-pool clearance. These advantages come at a certain price. Since peptides are monovalent, they tend to show decreased binding avidity compared to antibodies or $F(ab')_2$ fragments. Furthermore, the smaller size of the peptides favors their uptake in the proximal tubules of the kidney, as is the case with octreotide. This can be avoided in some cases by carefully designing the peptide or by administering lysine to block renal uptake (*30*).

Tumor Receptors

σ-Receptors. The biology of σ-opiate receptors is as yet incompletely understood; however, outside the brain and spinal cord, their expression has been detected in a wide variety of tissues, including heart, kidneys, adrenal glands, gonads, gastrointestinal tract, liver, and spleen. The receptors appear to be localized on the plasma membranes of subcellular organelles, such as Golgi bodies, endoplasmic reticulum, and nucleus, where they may be associated with G-proteins. Two subtypes, σ-1 and σ-2, have been identified.

σ-2 receptors are overexpressed in several tumor types, including melanoma, breast cancer, prostate cancer, and small cell cancer of the lung. In breast cancer, up to 1 million copies of the receptor per cell have been reported. Expression of σ-2 receptors correlates with proliferation status of tumor cells (higher in proliferating cells) (*31*). σ-1 receptors are also overexpressed in some tumors, including prostate cancer (*32*).

The drug pentazocine is a specific ligand for σ-1 receptors. 1,3-Di-2-tolyguanide has equal affinity for σ-1 and σ-2 receptors. Recently, Choi et al. synthesized a ^{99m}Tc-labeled σ-2–specific ligand, [*N*-[2-((3′-*N*′-propyl-[3,3,1]azabicyclononan-3α-yl)(2″-methoxy-5-methyl-phenylcarbamate)(2-mercaptoethyl)amino)acetyl]-2-aminoethanethiolato] technetium(V) oxide), and showed its uptake in mice implanted with mammary adenocarcinomas (*33*).

Breast Cancer Receptors. Hormonal therapy of breast cancer is well established. Approximately 55%–65% of primary breast cancers express estrogen receptors, and 45%–60% express progesterone receptors. For tumors expressing receptors, estrogen stimulation has a pro-proliferative effect. Tamoxifen and raloxifene are selective estrogen receptor modulators, which act as estrogen receptor agonists in some tissues (such as skeletal and cardiovascular tissues) and as antagonists in others (most importantly, in breast and breast tumors). Blocking of estrogen stimulation by either agent has been associated with decreased tumor growth and increased survival time in patients with breast cancer and decreased incidence of breast cancer in patients to whom therapy is given prophylactically. Radiolabeled estrogen analogs have been used to detect breast tumors in humans, to evaluate tumor estrogen receptor status, and to monitor response to antiestrogen therapy.

HER-2/neu is a transmembrane receptor tyrosine kinase. Overexpression is seen in 17%–30% of primary breast cancers and is a negative prognostic factor overall, although positivity is predictive of response to adjuvant doxorubicin treatment (*34,35*). In patients with estrogen receptor–positive tumors, HER-2/neu overexpression predicts a poorer response or apoptotic index in patients receiving anthracycline neoadjuvant therapy or tamoxifen therapy (*36,37*). Trastuzumab (herceptin), a new antitumoral agent, is a monoclonal antibody that binds the extracellular portion of HER-2/neu. Therapy with trastuzumab improves response as an adjuvant to first-line chemotherapy, but principally in patients with a high level of HER-2/neu overexpression. ^{131}I-Radiolabeled anti-HER-2/neu monoclonal antibodies have been synthesized and shown to have uptake in mammary tumors in mice (*38*). An ^{111}In-labeled form of the same antibody demonstrated uptake in human patients, and this uptake was a predictor of response to trastuzumab immunotherapy (*39*).

Cholecystokinin-B/Gastrin Receptors. The cholecystokinin-B (CCK-B)/gastrin receptor is widely expressed as a neurotransmitter in the telencephalon and as a gastrin receptor in the stomach. Its distribution is limited elsewhere in the body, although it is overexpressed in human pancreatic, gastric, and colorectal carcinomas as well as in some extraalimentary tract tumors, including small cell lung cancer, ovarian stromal tumors, and astrocytomas. It is also found in >90% of medullary thyroid carcinomas (*40*), and unlabeled pentagastrin injection has long been used as a provocative test for detecting primary, recurrent, or metastatic medullary thyroid carcinoma. ^{131}I-Labeled gastrin heptadecapeptide has been shown to accumulate in tumors in a patient with metastatic medullary carcinoma as well in nude mice with human medullary thyroid carcinoma xenografts (*41*). A

nonpeptide antagonist of the CCK-B receptor, 3R(+)-*N*-(2,3-dihydro-1-[^{11}C]methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-*N′*-(3-methylphenyl)urea, has also been synthesized (*42*). In mice, brain uptake of this agent was low, likely due to impermeability of the blood–brain barrier; therefore, without modification to increase its lipophilicity, this agent is unlikely to be useful as a tracer for brain CCK-B receptors.

Other Receptors. Various receptors for peptides and other small molecules have a high incidence of overexpression in breast cancer (*43*) and other tumor types. These include G-protein–coupled receptors, such as gastrin-releasing peptide receptor, vasoactive intestinal peptide receptor, and type 2 somatostatin receptor; tyrosine kinase receptors, such as platelet-derived growth factor (PDGF)-B receptor, vasoendothelial growth factor receptor (VEGF-R), insulin receptor, insulin-like growth factor type-1 receptor, epidermal growth factor receptor (EGF-R), fibroblast growth factor receptor types 4 and 1; and nontyrosine kinase receptors, such as leukemia inhibitory factor receptor and the β-subunit for the interleukin-2 receptor. In the wake of the success of trastuzumab in the treatment of breast cancer, efforts are underway to develop blocking antibodies and peptide antagonists against several other tumor receptors, including gastrin-releasing peptide receptor, EGF-R, PDGF, and VEGF. As is the case for trastuzumab, radiolabeled ligands have the potential to be useful in the selection and monitoring of therapy with these agents. Recent work on new receptor ligands have included ^{11}C-PD156707 and ^{18}F-BQ3020, radioligands for the endothelin receptor (*44*); ^{111}In-DOTA-lys8-vasotocin, an oxytocin analog (*45*); a ^{99m}Tc-labeled *N*4-functionalized stable neurotensin analog (*46*); and ^{123}I-*N*-(2-diethylaminoethyl)-2-iodobenzamide, a specific marker for melanoma (*47*).

Tumor Metabolism

Thymidine Phosphorylase. Thymidine phosphorylase is an enzyme that catalyzes the hydrolysis of thymidine to thymine and deoxyribose-1-phosphate. High levels of expression of thymidine phosphorylase have reportedly been associated with decreased survival in colorectal, head or neck, bladder, and cervical cancer and also with angiogenic activity of tumors (*48*). A specific inhibitor of the enzyme, 5-chloro-6-(2-iminopyrrolidin-1-yl)methyl-2,4(1*H*,3*H*)-pyrimidinedione, has been shown to shrink tumors in mice (*49*). Furthermore, since the enzyme also catalyzes the reverse reaction (i.e., conversion of thymine to thymidine), it can serve as a means of intracellular trapping of therapeutic analogs of thymine, such as capecitabine, which is converted to fluorouracil. Radiolabeled analogs could be used for cell killing, with ^{211}At, ^{125}I, or ^{131}I, or as imaging probes to identify tumors with high thymidine phosphorylase levels as candidates for therapy; to monitor response to treatment with antithymidine phosphorylase or other therapies; and to monitor tumor angiogenesis. Klecker et al. have synthesized several analogs and have demonstrated incorporation of one of these, bromouracil, into DNA in cultured cells expressing high levels of thymidine phosphorylase (*50*). Animal studies are pending.

Folic Acid Receptors. Membrane folic acid receptors mediate intracellular accumulation of folate and its analogs, such as methotrexate. Expression is limited in normal tissues, but receptors are overexpressed in various tumor cell types. Using ethylenedicysteine as a chelator, folic acid has been labeled with ^{99m}Tc, and tumoral uptake has been imaged in a rat breast-tumor xenograft model (*51*).

Tumor Hypoxia

Tissue hypoxia is central to the pathogenesis of cerebrovascular disease, ischemic heart disease, peripheral vascular disease, and inflammatory arthritis. It is also a ubiquitous feature of the growth of malignant solid tumors, where it bears a positive relationship to the aggressiveness of a tumor, and correlates negatively with the likelihood of response to chemotherapy or radiation therapy. Recent work has suggested that there is a common pathway of response to hypoxia in each of these settings. Hypoxia-inducible factor (HIF), a heterodimeric DNA transcription factor, appears to be the key player in this process. The β-subunit of HIF is a constitutive nuclear protein, which is not specific for hypoxia. The α-subunits are the specific hypoxia response elements. Under well-oxygenated conditions, they are rapidly degraded by the ubiquitin-proteasome pathway, in a manner that is dependent on interaction with the von Hippel–Lindau tumor suppressor protein (pVHL). In hypoxia, the subunits are stabilized and translocate to the nucleus, where they can dimerize with the β-subunit. Target genes for HIF are manifold and include several genes involved in tumor metabolism and proliferation: erythropoietin, transferrin and transferrin receptor, vascular endothelial growth factor and VEGF-R 1, glucose transporters 1 and 3, insulin-like growth factor–binding protein-1 and -3, insulin-like growth factor II, TGF-β3, and cyclin G2.

HIF-α subunits are not present in most normal human tissues but are highly expressed in many malignant tumors, particularly in areas adjacent to zones of necrosis. This pattern is seen in prostate, breast, lung, gastrointestinal tract, brain, ovary, melanoma, and mesothelioma. In clear-cell renal carcinoma and hemangioblastoma, expression is seen throughout the tumor, most likely related to loss of pVHL function rather than to hypoxia per se. In brain tumors, the degree of HIF-α expression correlates with tumor grade (*52*), as would be expected, since necrosis and hypoxia are histologic hallmarks of high-grade tumors.

To date, no imaging agents for hypoxia have directly targeted HIF. However, 2-nitroimidazole compounds are reduced and trapped in hypoxic cells and have been used in several studies as direct sensors of oxygen tension in ischemic myocardium and tumors. Examples include ^{18}F-fluoromisonidazole (FMISO) (*53*), ^{18}F-fluoroerythronitroimidazole (*54*), ^{123}I-iodoazomycin arabinoside (IAZA) (*55*), and ^{131}I-iodovinylmisonidazole (*56*). A more recent derivative,

^{18}F-fluoro-RP-170 (*57*), showed tumoral uptake in mouse squamous cell carcinoma and fibrosarcoma xenografts that correlated positively with that of ^{14}C-deoxyglucose and negatively with blood flow. The same compound demonstrated uptake in ischemic myocardium that was greater than that of ^{14}C-deoxyglucose but similar in pattern (*58*). Another agent, SR 4554 (*N*-(2-hydroxy-3,3,3-trifluoropropyl)-2-(2-nitro-1-imidazolyl) acetamide), has been designed for MRI applications but also has potential use in PET (*59*).

HL91 is a non-nitroimidazole compound that had tumoral uptake in a rat mammary tumor xenograft, which was maximal in tissue zones with morphologic criteria of hypoxia (perinecrotic areas or areas with cellular swelling and chromatin clumping) and correlated well with GLUT1 expression and uptake of ^{14}C-deoxyglucose (*60*). Other PET tracers in development include ^{64}Cu- and ^{60}Cu-labeled copper-diacetyl-bis(*N*4-methylthiosemicarbazone) (ATSM). These have shown uptake in ischemic myocardium in dog studies (*61*).

Tumor Proliferation

Uncontrolled cellular proliferation is one of the hallmarks of malignancy, and high histologic indices of mitotic activity are typically associated with increased cellular anaplasia and increased tumor aggressiveness. A proliferation-targeted imaging agent would potentially have high specificity for malignant tumors and could be used to differentiate benign or low-grade tumors from high-grade lesions, to detect high-grade transformation in a low-grade tumor, or to plan the optimal approach for diagnostic biopsy, surgical resection, or radiation therapy. As its increased degree of uptake correlates broadly with a higher grade of malignancy or biologic aggressiveness (*62*), ^{18}F-FDG is already in use for this purpose. However, FDG avidity bears only an indirect relationship to cell division, and uptake is not specific for tumors. ^{11}C-Methionine imaging suffers from similar drawbacks.

The search is thus under way for a more direct indicator of proliferation. While possible targets include proteins upregulated during the cell cycle (such as cyclins or DNA polymerase), most of the published work to date has focused on DNA analogs, which are incorporated into the replicated DNA strand. A prototype for this class of agents is ^{11}C-thymidine. This nucleoside, however, is rapidly metabolized, and the kinetics of its incorporation into DNA are too slow in comparison with the 20-min half-life of ^{11}C. Hence, ^{11}C-thymidine is unlikely to find widespread clinical application, and attention has focused on developing agents with longer half-lives and greater resistance to degradation. Bromodeoxyuridine (BrdU) is a thymidine analog used immunohistochemically to determine the labeling index, or fraction of cells in mitosis. A ^{76}Br-labeled form of this compound was introduced as a possible imaging agent (*63*). Subsequent studies have shown that a major portion of the tissue signal derives from free ^{76}Br in the extracellular fluid, not incorporated into DNA. In fact, the DNA incorporation rate has been reported to be as low as 9% of the total tissue activity (*64*), and attempts to boost this, using a diuresis protocol, have introduced only modest improvements. 5-^{76}Br-bromo-2′-fluoro-2′-deoxyuridine (^{76}Br-BFU) is stable to degradation and has been shown to have a much higher DNA incorporation rate—up to 80.5% in rat spleen (*65*). Uptake is limited by a very short plasma half-life; this can be improved by using cimetidine to inhibit renal elimination of the agent. High ratios of uptake in proliferating versus nonproliferating tissue have been demonstrated in a mouse fibrosarcoma model (*66*). Other nucleoside analogs under investigation include ^{18}F-1′-fluoro-5-(C-methyl)-1-β-D-arabinofuranosyluracil (FMAU) (*67*), ^{124}I-iododeoxyuridine (*68*), and ^{124}I-5-iodo-1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-uracil (FIAU) (*69*).

Fluorothymidine is yet another thymidine analog that has received substantial attention. Developed as a potential antileukemic and retroviral agent, it is a substrate for cytosolic thymidine kinase-1 (TK1), an enzyme of the nucleoside salvage pathway, which is expressed immediately before and during S-phase. Although <2% of the tissue activity of fluorothymidine is incorporated into DNA, phosphorylation by TK1 results in cellular trapping. In cell culture, uptake correlates with TK1 activity and cellular proliferation (*70*). In human PET studies with ^{18}F-3′-deoxy-3′-fluorothymidine, tumoral uptake values (standardized uptake values) have shown clear contrast with background activity in nonproliferating tissue and correlate well with Ki-67 scores, an immunohistochemical index of proliferative activity (*71*). However, liver and bone marrow activity is high, and uptake in tumors is generally less than that of ^{18}F-FDG (*72*). Given the increase in salvage pathway activity which typically accompanies cellular damage, this agent may have limited usefulness for assessing treatment response after chemotherapy or radiation therapy. However, it may have utility in some settings in tumor grading—for example, in brain tumors where background uptake of ^{18}F-FDG is high.

Apoptosis

Apoptosis, or programmed cell death, is an active, energy-dependent mechanism for the elimination of cells that have been injured, infected, or immunologically recognized as harmful or superfluous (*73*). While a great number of stimuli can initiate apoptosis, all ultimately lead to a common pathway, in which activation of a cascade of cysteine–aspartic acid proteases (caspases) leads to irreversible changes that include cytoskeletal disruption, chromatin clumping, internucleosomal DNA cleavage, and, ultimately, disintegration of the cell into small membrane-bound remnants targeted for rapid removal by macrophages. The process is rapid and can be accomplished without invoking an acute inflammatory response. Using terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling techniques, apoptosis has been demonstrated in a wide variety of physiologic processes, including fetal morphogenesis, postnatal brain remodeling, development of immune tolerance

via clonal deletion of T-cells, and turnover of senescent cells in the intestinal mucosa. It may also play a key role in the pathogenesis of some viral infections, transplant rejection, cardiomyopathy, periinfarct remodeling, and reperfusion injury. Apoptosis can be seen in a wide variety of malignant tumors, particularly in hypoxic zones adjoining areas of necrosis, and it is the endpoint of most forms of anticancer therapy.

As part of the apoptotic mechanism, phosphatidylserine, a phospholipid normally sequestered on the inner leaflet of the cell membrane, is abruptly translocated to the external leaflet. A 35-kDa endogenous human protein, annexin V, binds to exposed phosphatidylserine with an affinity in the nanomolar range. ^{99m}Tc-Annexin V was initially prepared by derivatization with hydrazinonicotinamide (*74*) and has been used in animal models to image apoptosis, as encountered in rejection of heart (*75*), lung (*76*), and liver (*77*) allografts; in arteriosclerotic plaques (*78*); and in neonatal rabbit brain after unilateral carotid artery ligation to induce hypoxia (*79*). In humans, increased annexin V uptake was shown in cardiac allograft recipients with histologic evidence of at least a moderate grade of transplant rejection (*80*). Annexin V uptake was also demonstrated in a small group of patients with acute myocardial infarction, and the region of uptake correlated well with the infarcted zone delineated by conventional perfusion imaging (*81*). Tumoral uptake of ^{99m}Tc-ethylenedicysteine–conjugated annexin V was also observed in breast cancer cells exposed in vitro to paclitaxel and 10–30 Gy of irradiation and in vivo in breast tumor–bearing rats treated with paclitaxel (*82*). While the degree of uptake of annexin V correlates well with the extent of cell death, uptake is not completely specific for apoptosis, as it can also be seen in cellular necrosis and in severe metabolic stress (*83*).

The apoptotic cascade is complex and offers several potential targets for imaging. Among these are the caspase enzymes, which are the key effectors. An inhibitor of caspase activity, benzyloxycarbonyl-Val-Ala-DL-Asp(*O*-methyl)-fluoromethyl ketone (Z-VAD-fmk), has been labeled with ^{131}I and investigated as a potential apoptosis imaging agent (*84*). In an in vitro model of Morris hepatoma cells transfected with the herpes simplex virus thymidine kinase (HSVtk) gene, a 2-fold increase in cellular uptake of Z-VAD-fmk occurred after induction of apoptosis with ganciclovir. Unfortunately, the absolute cellular uptake of ^{131}I-IZ-VAD-fmk was too low for in vivo imaging to be feasible without further modification of the agent.

Angiogenesis

In the absence of a mechanism for inducing angiogenesis, solid tumors are incapable of growing beyond submillimeter size. Inhibitors of angiogenesis are among the most promising new chemotherapeutic agents under investigation. Dozens of natural pro-angiogenic and antiangiogenic factors have been identified. The $\alpha_v\beta_3$ integrin is expressed on vascular endothelial cells during angiogenesis and vascular remodeling, particularly in pathways stimulated by VEGF (*85*). It is not expressed in mature vessels or in nonneoplastic epithelium. The integrin binds several ligands in the extracellular matrix, each containing the motif -Arg-Gly-Asp- (RGD). Disruption of this ligand interaction by competitive antibody binding has been shown to block formation of new blood vessels (*86–88*); this is the basis of a therapeutic monoclonal antibody, LM609, that is now in phase II trials. Cyclic peptides containing RGD sequences have high affinity and selectivity for $\alpha_v\beta_3$ integrin; these are also in clinical trials as therapeutic agents. Several studies have approached angiogenesis imaging using cyclic RGD peptides, labeled with ^{18}F (*89*), ^{99m}Tc (*90*), and ^{111}In (*91*), and high uptake has been shown in a mouse xenograft model of ovarian tumors (*92*). Should the clinical trials be successful, these agents could be useful for pretreatment confirmation of $\alpha_v\beta_3$ expression as well as for monitoring the response to therapy with $\alpha_v\beta_3$ antagonists.

Neuroreceptors and Neurotransmitters

Advances in neuropharmacology over the past several decades, particularly the development of highly specific ligands for receptors and transporters, have proven to be readily translatable to molecular imaging. Consequently, work in this field was begun early and has expanded apace, with the result that even a simple listing of agents under study is beyond the scope of this review. What follows is only a brief and impressionistic sketch of a few principal areas of interest.

Dopaminergic System. Loss of dopaminergic neurons in the substantia nigra is central to the pathophysiology of Parkinson's disease; however, this system projects widely to the forebrain cortex and limbic system, as well as to the striatum, and consequently appears to play a role in a variety of disorders other than Parkinson's disease, including schizophrenia, Tourette syndrome, autism, and drug addiction. The first agent to be introduced was ^{18}F-6-L-fluorodopa (*93*), a dopamine precursor analog that is a substrate for dopa decarboxylase. Its use for the direct assessment of dopaminergic function has been well validated, and in clinical studies it is able to distinguish Parkinson's disease from vascular encephalopathy (lower body parkinsonism) and to aid in early diagnosis. Similar results have been obtained with agents targeting the dopamine reuptake transporter, particularly the tropane family, derived from cocaine. The SPECT agent 2β-carbomethoxy-3β-(4-^{123}I-iodophenyl)tropane (β-CIT) (*94*) is a widely investigated representative of this group that is in clinical use in Europe. Related derivatives include ^{18}F-FE-CNT (*95*), ^{76}Br-FE-CBT (*96*), *N*-(3-iodopropen-2-yl)-2β-carbomethoxy-3β-(4-chlorophenyl) tropane (^{123}I-IPT) (*97*), and 2β-carbomethoxy-3β-(4-^{18}F-fluoromethylphenyl)tropane (p-FWIN) (*98*).

At least 5 dopamine receptor subtypes have been identified to date, which can be classified as D_1- or D_2-like, based on their activation or inhibition of adenylate cyclase, respectively. Postsynaptic D_2 receptor binding on GABAergic neu-

rons in the striatum is generally little changed in Parkinson's disease but is decreased in atypical parkinsonian syndromes such as progressive supranuclear palsy or multiple system atrophy, so receptor imaging agents are able to distinguish these etiologies. Representative ligands for D_2 receptors include ^{11}C-raclopride (*99*) and ^{123}I-s(−)iodobenzamide (IBZM) (*100*). D_1 receptor ligands include ^{11}C-SCH 23390 (*101*) and ^{11}C-NNC-112, ^{11}C-NNC-687, and ^{11}C-NNC-756 (*102*) as well as the SPECT agent (+)-1-(2-^{123}I-iodo-4,5-dimethoxybenzyl)-7-hydroxy-6-methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline (A-69024) (*103*).

Serotoninergic System. Serotoninergic neurons in the brain stem have widespread projections throughout the cerebral cortex, limbic system, and thalamus, and dysfunction of this system has been implicated in a correspondingly wide variety of disorders, including depression, bulimia nervosa, autism, and chronic anxiety syndromes. A significant role in schizophrenia and Alzheimer's disease (AD) has also been suggested. Like dopamine, serotonin is a substrate for a specific reuptake transporter. An initial agent for PET imaging of the serotonin transporter, ^{11}C-(+)-McN5652 (*104*), found its way into human trials; however, slow uptake and high nonspecific binding have led to a search for a more suitable agent. A large number of phenylthiophenyl derivates are under study, including the SPECT agents 403U76 (*105*), IDAM (*106*), and ADAM (*107*), and the ^{11}C agents DASB and DAPP (*108*). Recently, synthesis of an ^{18}F-labeled agent, 2-[(2-amino-4-chloro-5-fluorophenyl)thio]-*N*,*N*,-dimethyl-benzenmethanamine (ACF), has been reported (*109*). The tropane ^{11}C-labeled 3β-4′-isopropenyl-phenyl)nortropane-2β-carboxylic acid methyl ester (RTI-357) also shows specific uptake by the serotonin transporter (*110*).

At least 7 major classes of serotonin (5-hydroxytryptophan) receptors have been identified to date, some of which have several subtypes. 5-HT_{1A} receptors are particularly prominent in the limbic system. Of the many ligands under study, most are derivatives of *N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridyl)cyclohexanecarboxamide (WAY-100635) (*111*), a receptor antagonist; of 8-hydroxy-*N*,*N*-(dipropylamino)tetralin (8-OH-DPAT) (*112*), a receptor agonist; or of apomorphine. 5-HT_{2A} receptor ligands include the PET agents ^{11}C-methylspiperone (*113*), ^{18}F-altanserin (*114*), and ^{18}F-setoperone (*115*) and the SPECT agents ^{123}I-ketanserin (*116*) and ^{123}I-5-I-R91150 (*117*).

Cholinergic System. Postsynaptic muscarinic acetylcholine receptors are widely expressed in the cerebral cortex and basal ganglia. There is evidence for a key role of this system in learning and memory, and abnormalities of muscarinic neurotransmission have been described in AD and Pick's disease as well as in schizophrenia. Studied ligands include [*N*-^{11}C-methyl]benztropine (*118*), ^{123}I-IPIP (*119*), and (*R*,*S*)-3-quinuclidinyl-4-^{123}I-iodobenzilate (IQNB) (*120*). Neuronal nicotinic receptors have a more restricted distribution but are found in sites known to play roles in cognition and memory, emotion, and control of movement, including the thalamus, hippocampus, striatum, and hypothalamus. Not surprisingly, the nicotinic system has been implicated in the pathophysiology of addiction, depression, anxiety, schizophrenia, AD, and Parkinson's disease, and loss of cortical nicotinic acetylcholine receptors has been shown to correlate with the severity of clinical deficits in AD. Representative ligands include ^{11}C-nicotine (*121*) and 5-^{123}I-iodo-A-85380 (*122*).

The observation of decreased neocortical and hippocampal acetylcholinesterase activity in AD has provided a rationale for therapy with cholinesterase inhibitors. Acetylcholinesterase activity itself has been evaluated with ^{11}C-physostigmine (*123*) and, more recently, with *N*-^{18}F-fluorethyl-4-piperidyl acetate (FetP4A) (*124*). Yet another approach to cholinergic imaging targets the acetylcholine vesicular transporter, using agents such as (−)-5-^{123}I-iodobenzovesamicol (IBVM) (*125*).

Glutamatergic System. Glutamate is a ubiquitous, excitatory neurotransmitter, with receptors on virtually all neurons within the brain. Six major receptor families have been identified. Among these, the voltage-gated calcium channels known as NMDA receptors (named for a specific pharmacologic agonist, *N*-methyl-D-aspartate) have been the best studied. This is in itself a complex group, with 2 major subtypes, each with multiple isoforms or splicing variants. Receptor response is modulated by binding sites for ketamine and phencyclidine (PCP). The density of these receptors is greatest in the pyramidal neurons of the hippocampus, consistent with their presumed key role in learning and memory. Hyperstimulation of NMDA receptors is also believed to be a major mechanism for neuronal damage in epilepsy and cerebral ischemia. A prototypical imaging ligand is *N*-(1-naphthyl)-*N*′-(3-^{125}I-iodophenyl)-*N*′-methylguanidine (^{125}I-CNS 1261) (*126*). Baseline uptake of this agent in rat brain has been shown to be markedly increased in ischemia (*127*).

Opioid System. The best-studied members of the opioid receptor family are the μ-subtype (most specific for binding opioid analgesics) and the δ- and κ-subtypes (relatively specific for enkephalins and dynorphins, respectively). Apart from their obvious role in pain control and addiction, opioid receptors have been implicated in Parkinson's disease, Huntington's disease, and AD as well as in seizure disorders. ^{11}C-Carfentanil is a μ-specific ligand that has been extensively studied (*128*). ^{11}C-Methyl naltrindole is a δ-specific agent (*129*), while ^{18}F-cyclofoxy binds both μ- and κ-receptors (*130*). Diprenorphine is a well-studied but less specific agent, derivatives of which have been labeled with ^{11}C (*131*), ^{18}F (*132*), and ^{123}I (*133*).

Abnormal Proteins in Brain

AD is the prototype of a complex group of primary neurodegenerative disorders, affecting 4 million sufferers in the United States and estimated to affect 14 million by the year 2050, in large part due to the aging of the population. Over 90 billion dollars is spent annually on AD-related expenses in the United States alone. Imaging methods based

on patterns of cerebral perfusion have shown significant utility in the diagnosis and monitoring of these patients; however, an intensive search is under way for agents showing improved specificity. The distinctive lesions of AD include neurofibrillary tangles, largely composed of a phosphorylated form of tau, a neuronal microtubule-associated protein, and senile plaques, containing β-amyloid as a major component. Most experimental approaches have been directed toward one or the other of these 2 constituents.

Chrysamine G is an analog of the dye Congo red, which has long been exploited in histochemical studies for its apple-green birefringence, seen after intercalation of the dye into the amyloid fibrils. Radiolabeled chrysamine G (^{99m}Tc-MAMA-CG) localized to cortical amyloid deposits in autoradiographic studies of human brain at autopsy, and uptake into mouse brain has been shown in vivo (*134*). Other amyloid-specific agents include 10H3, a ^{99m}Tc-labeled monoclonal antibody targeting residues 1–28 of the β-amyloid protein (*135*), and ^{123}I-labeled serum amyloid P component (*136*). Basic fibroblast growth factor (bFGF) is expressed at high levels in neurons adjacent to plaques, possibly as a result of vascular perfusion abnormalities, and radioiodinated bFGF has shown amyloid-specific uptake in transgenic mouse brain after intranasal administration (*137*). While these agents have good binding characteristics in vitro, the principal obstacle in vivo has been poor penetration of the blood–brain barrier. Some newer classes of agents have shown better performance in this regard. These include 2-(4′-dimethylaminophenyl)-6-iodobenzoxazole (IBOX), a thioflavin derivative (*138*); 2-(1-(6-[2-^{18}F-fluoroethyl) (methyl)amino]-2-naphthyl)ethylidene)malononitrile (FDDNP), a napthalene derivative that labels plaques and tangles (*139*); and (*trans,trans*)-1-bromo-2,5-bis(3-hydroxycarbonyl-4-hydroxy)styrylbenzene (BSB), which binds β-pleated sheets found in amyloid, neurofibrillary tangles, and Lewy bodies (*140*).

Response to Infection

An ideal imaging agent would provide rapid localization to an infectious focus, with sufficient specificity to distinguish pyogenic infection from aseptic inflammation. The earliest agents to adopt a molecular targeting rationale were antigranulocyte antibodies. Antigens targeted have included NCA-90 (CD66c), expressed on activated neutrophils (*141*), as well as NCA-95 (*142*) and CD15 (SSEA-1) (*143*). Most of these preparations have been labeled with ^{99m}Tc or ^{111}In, using a DTPA or hydrazinonicotinamide (HYNIC) linker. IgG, IgM, and F(ab)′ fragments have all been used. While many of these agents have shown a sensitivity comparable to ^{111}In-labeled white blood cells, much of the uptake appears to be due to nonspecific factors, such as increased vascular permeability, rather than to specific labeling of circulating granulocytes. Indeed, few radiopharmaceuticals have been shown to have any clear advantage over nonspecific polyclonal human IgG (*144,145*).

Several peptide agents have been investigated. Chemotactic peptides bind receptors on granulocytes in acute inflammation, and agents such as radiolabeled formyl-Met-Leu-Phe and its derivatives have shown superior localization to infected sites compared with ^{111}In-labeled white blood cells (*146*). Unfortunately, even at imaging doses, these agents are capable of inducing marked granulocytopenia, which has precluded their clinical use. Similar potent biologic effects have hindered the introduction of agents targeting receptors for complement anaphylatoxin C5a, interleukin-1 and -8 (for acute inflammation), and interleukin-2 (for chronic inflammation), although imaging characteristics have been promising (*147–149*). Other targets currently under study include bacterial receptors for cationic peptides derived from human ubiquicidine (*150*) and the HLA class-II-like antigen (li determinant), expressed on B lymphocytes and monocytes (*151*).

Multiple Drug Resistance

P-glycoprotein (Pgp), the product of the multidrug resistance gene MDR1, is a prototype of a class of energy-dependent efflux transporters for lipophilic cations, which are found in a variety of normal tissues, including capillary endothelial cells of the brain, the biliary canalicular surface of hepatocytes, and the proximal tubule cells in the kidney. Overexpression of Pgp is also seen in several malignant tumor types, where it limits the effectiveness of a wide variety of chemotherapeutic agents, such as daunorubicin, vincristine, etoposide, and adriamycin, which are substrates for its activity. The myocardial perfusion agents ^{99m}Tc-hexakis(2-methoxyisobutylisonitrile)technetium(I) (sestamibi), ^{99m}Tc-tetrofosmin, and ^{99m}Tc-furifosmin (Q12) are also lipophilic cations and, as such, are substrates for Pgp and other multidrug resistance genes wherever they are expressed. Although the initial uptake and concentration of these agents is due to nonspecific factors such as perfusion or electrostatic interactions at the mitochondrial membrane, in tumors the washout kinetics will reflect the level of expression of MDR1 or cognate multidrug resistance genes (*152*). In this context, a conventional physiologic agent such as sestamibi can function as a more-or-less specific molecular targeting agent. Assessment of the level of activity of Pgp can thus potentially predict the response to chemotherapy (*153*) or indicate the need for adjunct therapy with inhibitors of Pgp, such as verapamil or cyclosporin, or the newer agents PSC 833, GF120918, or VX-710. Apart from the SPECT agents described, several PET agents are under investigation, including ^{11}C-colchicine (*154*), ^{11}C-verapamil (*155*), ^{11}C-daunorubicin (*156*), and *N*-^{11}C-acetyl-leukotriene E4 (*157*).

Reporter Genes

A reporter gene is an exogenous gene that has been artificially inserted into a targeted cell or tissue and whose product has an activity that is readily distinguishable from that of native genes within the cell. In laboratory studies with transgenic animals, such reporters are often fused with promoters of native genes to provide a vicarious estimate of the activity of transcriptional modulators or inducers of

gene expression. However, in the clinical setting, reporter genes cointroduced with a gene therapy agent could be used as a convenient way to confirm successful introduction and expression of the therapeutic gene. Most of the imaging approaches to date have used reporter genes derived either from the dopamine type 2 receptor (*157*) or the thymidine kinase of the type 1 herpes simplex virus (HSV-TK1) (*158,159*). Targeting agents for HSV-TK1 have included ^{18}F-fluoroganciclovir (FGCV) (*160*), ^{18}F-fluoropenciclovir (FPCV) (*159*), ^{124}I-FIAU (*67*), and 9-[4-^{18}F-fluoro-3-(hydroxymethyl)butyl]guanine (FHBG) (*161*), among others.

TARGETED RADIONUCLIDE THERAPY

A new generation of labeled antibody agents is being developed for therapeutic purposes. The major barriers to the effectiveness of the existing ones, especially against solid tumors, are extended residence in the bloodstream, resulting in a high marrow dose, and poor penetration into some parts of the tumor, resulting in a nonuniform distribution of absorbed radiation dose. To circumnavigate these drawbacks, antibody fragments (Fab, $F(ab')_2$) or genetically engineered entities (F_v) have been used in an attempt to shorten the residence time and improve penetration into and attendant uniformity within tumors (*162*). Alternatively, the use of pretargeting with unlabeled antibody or antibody fragments containing an additional affinity site (e.g., strepavidin) followed by a chase of a small, rapidly excreted radiolabeled molecular conjugate (e.g., radiobiotin) can be used (*163,164*). Often, this strategy includes the use of an unlabeled conjugate to clear the blood of any residual circulating antibody.

Recently, the possibility of using α-particle emitters as effector radionuclides in radioimmunotherapy has been introduced (*165*). The high–linear-energy-transfer radiation produced is lethal with one α-particle-track traversal of the cell nucleus. Whether normal tissues can be spared with such therapy remains the issue.

Several new technologies are in earlier stages of development. Antisense agents targeted at upregulated oncogenes, either at ones that are amplified or at their mRNAs, are being synthesized (*166–168*). These can be triplex-forming oligonucleotides against DNA or protease-resistant anti-mRNA. A third species, less well developed, are aptamers or nucleic acid entities that have an affinity to intracellular signaling proteins. Considerable technologic difficulties need to be overcome before this group of therapeutic agents becomes a reality but, because of their potential specificity, the promise is great.

Another group of potential radiotherapeutic agents are those labeled with Auger electrical-cascade emitters (*169*). Because of their ultra-short range (nm) and high ionization density, these radionuclides are attractive as radiotherapeutic agents when they are uniformly taken up in the cell nuclei of tumors. In experimental systems, radiolabeled DNA precursors, intercalating agents, hormones with DNA receptors, groove-binding small molecules, and triplex-forming oligonucleotides are being explored. Labeled principally with ^{125}I or ^{123}I, these agents have been shown to be effective against experimental malignant ascites and brain and spinal cord tumors. Locoregional application of the DNA precursor 5-^{125}I-iodo-2′-deoxyuridine shows particular promise in neoplastic meningitis when administered intrathecally (*170*). The molecule is dehalogenated on entering the circulation, rapidly excreted, and thus exhibits little to no toxicity, even at high doses. The therapeutic effectiveness of Auger-emitting radionuclides when confined to the nucleus is due to the production of extensive DNA fragmentation that is difficult to repair.

Radiolabeled prodrugs that can be enzymatically converted to radiotherapeutic agents trapped within cancer cells or precipitated in their environment are in an early stage of development. The enzymes can be the products of upregulated oncogenes or can be introduced by gene therapy. An advantage of the precipitate method is that it is not necessary that all cells of the tumor express the converting enzyme. In any event, as molecular targeting for diagnosis becomes more sophisticated, its use for radionuclide therapy is also likely to do so.

In boron neutron capture therapy, the targeting agent is conjugated with a compound containing ^{10}B, a stable nuclide with a large neutron capture cross-section. After the pharmaceutical has had time to localize, the target tissue is irradiated with slow neutrons. In the resulting neutron capture reaction ($^{10}B(^{1}n,^{4}He)^{7}Li$), high-energy $^{4}He^{2+}$ and $^{7}Li^{3+}$ ions are produced with cytocidal effect. This approach has been widely studied in the treatment of gliomas and malignant melanoma, often using relatively nonspecific agents such as sulfhydryl boron hydride and boronophenylalanine. More recently, this technique has been combined with a molecular targeting approach using monoclonal antibodies direct against tumor antigens or ligands for EGF-Rs (*171*) and folate receptors (*172*).

WHERE WILL FUTURE WORK BE FOCUSED?

That there remain major technical challenges to molecular targeting should be evident from the foregoing review. In general, these are related to the rapid and specific delivery of tracer to the target, the elimination of unwanted background activity, and the production of sufficient signal to form a useful image or, in the case of therapeutic agents, to create a cytocidal effect. To a great extent, further development of this field will be determined by success in solving these challenges.

Improved Delivery

By rapidly concentrating the tracer at the site where the target is most available, unwanted background activity can be reduced, and maximum signal can be obtained for a given dose of injected radioactivity. Most of the agents developed so far are injected directly into the bloodstream, with an initial distribution determined by free diffusion

throughout the entire vascular and interstitial fluid space. This is perhaps adequate for targets that are readily accessible at the cell surface; however, more sophisticated delivery systems will be required to reach targets within the cell or the nucleus. Possible gateways to be explored would include pinocytosis, membrane fusion, and receptor-mediated endocytosis. The most efficient delivery systems would use pretargeting, with the tracer agent being directed toward the cell population or subcellular compartment where the target is most likely to be concentrated. This could use a single injection of a complex delivery vehicle or sequential injections of separate pretargeting and tracer components. This technology has already received considerable attention in the context of gene therapy, with liposomes (*173*), ligand-conjugated polyethyleneimine complexes (*174*), and viral-derived agents (particularly adenoviruses and retroviruses) being the most successful vehicles to date (*175,176*).

Improved Kinetics

The kinetics of uptake and elimination of unbound tracer must necessarily be shorter than the off-rate of the tracer when it binds to the target. They must also be shorter than the physical half-life of the labeling radionuclide. Thus, as a rule, agents with faster kinetics are superior to those with slower kinetics. Some agents, such as antibodies, are eliminated too slowly for use with any of the PET agents now in common application. Improvements in this area will tend to focus on downsizing agents (e.g., from antibodies to $F(ab')_2$ fragments to peptides) or using natural pathways of metabolic degradation and elimination (such as enhanced diuresis of ^{76}Br-BrdU). More aggressive approaches could potentially use plasmapheresis or dialysis to rapidly eliminate unbound tracer or delivery vehicles.

Signal Multiplication

As explained above, receptor and antibody agents depend on a receptor–ligand interaction in which there is rarely more than a single molecule of tracer bound to the target. Even with maximal specific activity of labeling of the tracer, the signal from rare targets may be too faint to detect. The signal can be boosted, however, if the binding ligand is joined to a catalytic functional group and a radiolabeled substrate for this catalytic moiety is then administered to produce the actual signal. Such bifunctional, hybrid agents would also considerably enhance the cytocidal effect of radiotherapeutic agents. Substantial development along these lines will be essential if molecular targeting is to achieve anything more than a down payment on its promised rewards.

Modular Design of Tracers

Nearly all of the agents described above have been designed as integral molecular formulations. This means that for each agent, the problems of delivery, target binding, and efficient radiolabeling have needed to be addressed more or less de novo. The effect is a bit like having to reinvent hydraulic brakes for each model of automobile on the road. A more efficient approach would be to design separate components for each function, which could then be integrated with each other by means of linkers, such as streptavidin–biotin couples or hybridizing sequences of DNA. This trend is already emerging and is likely to become commonplace in the future.

Tracer Batteries

As described above, pathotypes are complex profiles of the expression of multiple gene products. Full characterization of a disease state may, therefore, need to evaluate each of these several targets. For example, pretherapeutic workup of a tumor may require an assessment of proliferation, hypoxia, and angiogenesis, using a battery of agents, administered either sequentially or simultaneously (if labeled with radionuclides with distinct photopeaks of emission). Much of the power of molecular targeting will emerge from the ability to customize such panels of agents to address the needs of an individual patient.

WHAT ARE THE LIMITS OF MOLECULAR TARGETING?

Molecular targeting is in its infancy. The scope of the work cited in the preceding sections attests to the enthusiasm now prevalent in the field, and the achievements to date do much to justify that enthusiasm. But, inevitably, some constraints will be encountered. These include limitations imposed by the nature of the pathologic process, the design and labeling of probes, the process of delivering probes to the target, and the nature of the probe emissions used for detection or cell killing. Molecular imaging can only be as good as the imaging technology on which it is based.

The ultimate sensitivity of any agent is limited by the type of signal emitted and the detection system for that signal. Methods based on radioactive decay emissions are at present at least an order of magnitude more sensitive than any other method in clinical use, such as MRI; however, the maximum achievable sensitivity is limited by several factors, including the type of decay, the decay constant, the photopeak or particle energy of the emission, the specific activity of the probe labeling, detector characteristics, and, in a clinical setting, the acceptable duration of image acquisition. Even when all of these parameters have been optimized, biologic factors such as the mass of tissue expressing the target, the absolute number of target molecules expressed per cell, the receptor binding affinity or catalytic turnover rate for a target, the competition with endogenous ligands or substrates, and the metabolism and excretion of the probe will still limit signal from many candidate targets below the threshold of detection. Obviously, many molecules with a very low copy number per cell will never be feasible targets for detection, even by an ideally constructed probe.

Specificity, however, may ultimately turn out to be more of a limiting factor than sensitivity, and it is certain to assume more and more significance as the ultimate sensitivity is achieved. For one thing, the delivery of injected

agents is highly nonspecific. Uptake of the probe by the target will always be in an equilibrium with the overall volume of distribution, and there will always be some degree of misdelivery, through cross-recognition of the probe by related transporters or enzymes, through bulk processes such as lipophilic diffusion and renal filtration, or through sequestration by binding to plasma proteins and other macromolecules. These problems do not stop at the cellular plasma membrane, inasmuch as analogous processes are involved in translocation of the probe from one subcellular compartment to another. Even probes aimed at targets at the cell surface will show nonspecific intracellular uptake via endocytosis and sequestration in lysosomes.

Further challenges to specificity arise from the nature of the pathologic process itself. With the conspicuous exception of molecules of exogenous microbial origin, most targets of interest are likely to show, at best, a quantitative increase in expression in diseased tissue. There will always be some constitutive background level of expression, and where the focus of diseased tissue is very small or sparsely distributed, the relative changes in probe uptake may be too subtle for detection. Even when a signal is detected, it may be ambiguous. Cellular responses to injury use many common pathways, which amount to an overlapping of pathotypes. While the total profile of gene expression may be specific, very few individual molecules will be restricted to a single pathologic process. Most will be shared among multiple pathotypes. For example, uptake of FDG is increased in both malignant tumors and inflammation. While one may minimize the ambiguity by using batteries of probes or addressing targets of greater specificity, this only serves to lessen the problem, not eliminate it. Thus, it will always be necessary to interpret the study in a clinical context, and this will always require the oversight of an experienced clinician.

Some of the most attractive targets may exhibit such a range of pleomorphism that no universally applicable probe can be generated. This is particularly true for the detection of genetic mutations. Many premalignant precursor lesions exhibit chromosomal rearrangements or gain-or-loss-of-function mutations of cellular protooncogenes, similar to those of fully evolved malignant tumors. Detection of these would be highly beneficial, as it would permit preemptive treatment before malignancy has developed. Unfortunately, with rare exceptions, these mutations occur randomly among many possible sites in a variety of possible genes. The idiosyncrasy of the process largely precludes the construction of a probe that would have any general applicability as a screening device.

While a sober evaluation of limiting principles is useful to avoid wastage of effort, it need not curb the enthusiasm for this new field, which is fully justified by the possibilities and by the results already achieved. Conventional anatomic imaging has limitations too, but it transformed the practice of medicine within the boundaries of these limits. So too is molecular targeting likely to transform our practice in the clinic, to the extent that tests and treatments that scarcely seem conceivable now will be routine in a few years.

ACKNOWLEDGMENTS

The authors acknowledge the generous help of Alan J. Fischman, Victor H. Gerbaudo, and S. Ted Treves in reviewing this manuscript.

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