Oncogenes, Cancer and Imaging

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At the dawn of the 21st century, nuclear oncology is undergoing a formidable and rapid mutagenesis. The progress in radiochemistry, radiopharmacy and, foremost, the advances in molecular oncology are the determinant mutagenic factors. Mutation, amplification, deletion or translocation of deoxyribonucleic acid segments in proto-oncogenes and tumor suppressor genes also called anti-oncogenes account for the uncontrolled cell growth and proliferation resulting in cancer. The astonishing developments in peptide and nucleic acid chemistry have opened the door for the development of new, highly specific probes such as antisense, aptamer and peptidomimetic molecules to image the oncogenes and anti-oncogenes transcriptional (messenger ribonucleic acid) and translational (protein) products involved in carcinogenesis. In this article, I shall review the basic molecular mechanisms of carcinogenesis and describe the molecular probes that are currently being developed.

Key Words: oncogenes; tumor-suppressor genes; molecular probes

J Nucl Med 1999; 40:498–504

A gene is made up of a deoxyribonucleic acid (DNA) segment in the cell nucleus chromosome that specifies the amino acid sequence of a particular protein. Of the 50,000–100,000 genes in our genome, about 100 have been identified thus far as proto-oncogenes and about a dozen as tumor suppressor genes (Table 1). Over the past two decades, extensive biological analyses have established that the protein products of proto-oncogenes and tumor suppressor genes are part of a stringently regulated intra- and extracellular signaling network that choreographs the intricate sequences by which a cell enlarges and divides. Proto-oncogenes encourage cell growth and division. They code for four classes of oncoproteins that stimulate cell division (Fig. 1). Growth factors, such as the so-called platelet-derived growth factor and the fibroblast growth factor (FGF-5), are members of the first class. Growth factor receptors, such as the epidermal growth factor family of receptors ([EGFR] *erbB-2, erbB-3* and *erbB-4)* and the angiotensin receptors, are examples of the second class of oncoproteins. Many proto-oncogenes code for proteins involved in the membrane-associated cytoplasmic signal transduction pathway. This third class of oncoproteins is the most abundant and includes various tyrosine and serine/threonine kinases, several guanosine triphosphate binding proteins activated by cytokines and G protein receptors and other signaling cytoplasmic proteins. Nuclear transcription factors form the fourth group. These factors, on activation, often by phosphorylation, increase the expression of target genes by binding to DNA and activating transcription. Ultimately, the activation of these genes provides the cell resources and fuels to grow and divide.

In contrast to proto-oncogenes, the products of tumor suppressor genes provide signals that tightly control cell growth and proliferation. Although the effects and precise action mechanisms of these genes are poorly understood, few genes discovered in any field of research have achieved higher notoriety than the p53 tumor suppressor gene. This is because p53 is more frequently mutated in human cancers than any other known gene. Most of the current knowledge on the action of tumor suppressor genes is derived from the elucidation of the mechanisms of action of p53. p53 is a transcription factor that binds to a DNA sequence on chromosome 21 and activates the transcription of the *WAF1* gene (Fig. 2). The ensuing protein product (the *WAF1* protein) inhibits the function of members of a protein family called the cyclin-dependent kinases (Cdks). Cdks are the master controllers of cell cycle progression. In fact, *WAF1* is a potent inhibitor of the Cdk-cyclin complexes required for the transition from the G1 to S phases of the cell cycle, as well as for progression through S phase. Although growth suppression is believed to represent the major biological function of p53, in certain circumstances, p53 is also capable of inducing a certain type of programmed cell death called apoptosis. The Rb gene also plays a critical role in cell growth (Fig. 2). In fact, the underphosphorylated Rb protein (pRb) is the master brake of the cell cycle. Most current evidence suggests that pRb binds to and regulates the activity of transcription factors, particularly the E2F protein that activates the promoters of several genes required for DNA synthesis. The pRb-E2F complex also appears to downregulate numerous G1 exit-promoting protein genes, including c-*myc* and c-*myb*. Phosphorylation of Rb by various cyclins releases free E2F, which then interacts with the S-phase gene promoters. Apoptosis and growth suppression are part of the same cellular deterministic mechanism to prevent the propagation of cells harboring gene mutations.

Received Mar. 13, 1998; revision accepted Oct. 7, 1998.

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TABLE 1
Representative Oncogenes in Human Tumors

Oncogene	Tumor associations	Mechanism of action	Gene product
APL-RARa	Acute promyelocytic leukemia	Chromosomal translocation	Membrane protein
bcl-2	Follicular and undifferentiated lymphoma	Chromosomal translocation	Membrane protein
erbB	Mammary carcinoma, gliobastoma	Amplification, rearrangement	Growth factor receptor
erbB-2	Mammary, ovarian and stomach carcinoma	Amplification	Growth factor receptor
Cdk4	Sarcoma	Amplification	Cyclin-dependent kinase
gsp	Pituitary tumor	Point mutation	Signal transducer
hst	Stomach carcinoma	Rearrangement	Growth factor like
MDM2	Sarcoma	Amplification	p53 binding protein
myc	Lymphoma, carcinoma	Chromosomal translocation, amplification	Nuclear transcription factor
L-*myc*	Small cell lung carcinoma	Amplification	Nuclear transcription factor
N-*myc*	Neuroblastoma	Amplification	Nuclear transcription factor
raf	Stomach carcinoma	Rearrangement	Serine/threonine kinase
H-*ras*	Bladder carcinoma	Point mutation	p21 GTPase
K-*ras*	Lung, colon, pancreas carcinoma, leukemia	Point mutation	Signal transducer
N-*ras*	Leukemia	Point mutation	Signal transducer
ret	Papillary thyroid carcinoma	Rearrangement	Cell surface receptor
trk	Papillary thyroid carcinoma	Rearrangement	Growth factor receptor
ttg	T-cell acute lymphoid leukemia	Chromosomal translocation	Transcription factor

ROLE OF ONCOGENES AND TUMOR SUPPRESSOR GENES IN CARCINOGENESIS

Studies initiated to understand the molecular mechanisms associated with the cancerous phenotype have demonstrated that alterations in the genes that participate in the signal transduction process result in specific genetic lesions that derail cells from normal to uncontrolled growth. These quantitative and/or qualitative alterations are responsible for both the activation of growth-promoting genes or oncogenes and the inactivation of growth-constraining genes, also called tumor-suppressor genes or antioncogenes.

Both structural and regulatory alterations have been found to account for the activation of cellular proto-oncogenes and determine a cell to grow autonomously. These alterations are represented in Figure 3. Point mutations are the simplest structural alterations that a DNA sequence can undergo. They consist in single base DNA changes resulting in modification of the amino acid sequence of the encoded protein. Most often, point mutations confer a loss of function to the affected protein. However, they can also confer active properties to the oncoprotein (Fig. 4A). Regulatory changes are produced by translocation or amplification of a chromo-

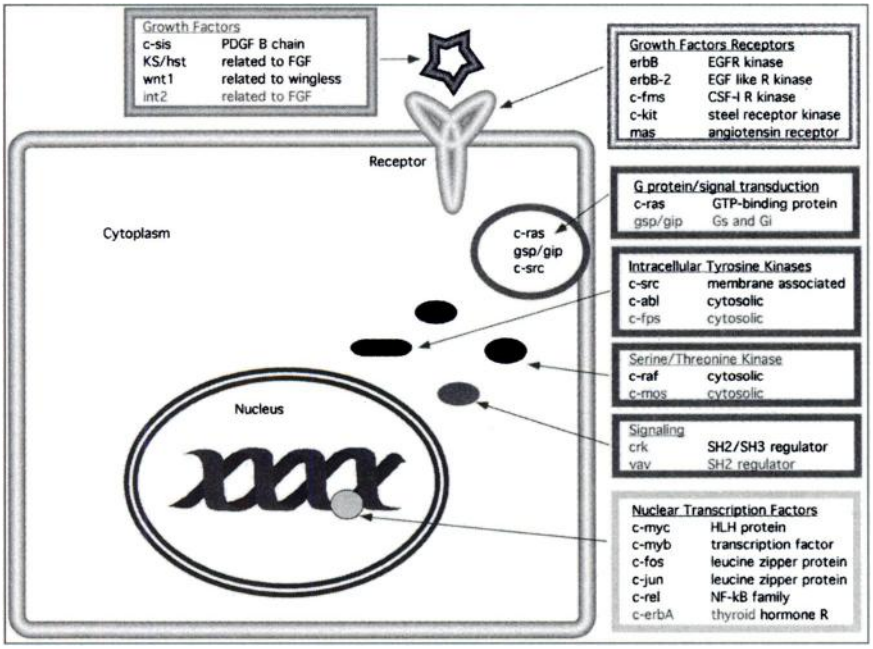

FIGURE 1. Oncogenic proteins intervene at different level of cell growth and differentiation.

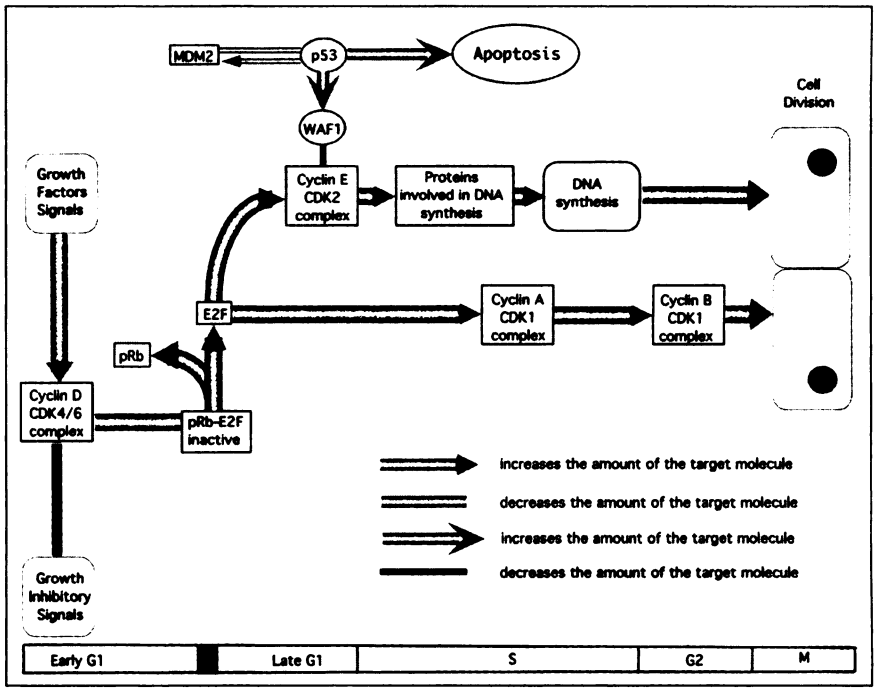

FIGURE 2. Role of growth and inhibitory factors and p53 on cell growth and division.

somal segment carrying a proto-oncogene. The translocation mechanism juxtaposes a proto-oncogene and an unrelated regulatory region and determines the deregulated synthesis of a normal protein. The amplification mechanism leads to a deregulated replication of a proto-oncogene and multiple copies of the gene appear either as tandemly duplicated segments within the chromosome or as extrachromosomal particles. In both instances, an increased amount of the messenger ribonucleic acid (mRNA) and its protein product ensues.

Tumor suppressor genes are part of a group of genes acquired at conception that determine the probability that cancer will develop in an individual. These "susceptibility" genes function at different levels of cell metabolism and growth. For example, they regulate the metabolism of carcinogenic compounds, recognize and repair DNA damages and recognize and eradicate tumoral antigens. In contrast to proto-oncogenes, which require activation to induce cancer, it is the inactivation of both alleles of tumor-suppressor genes that triggers cancer. Both mutations

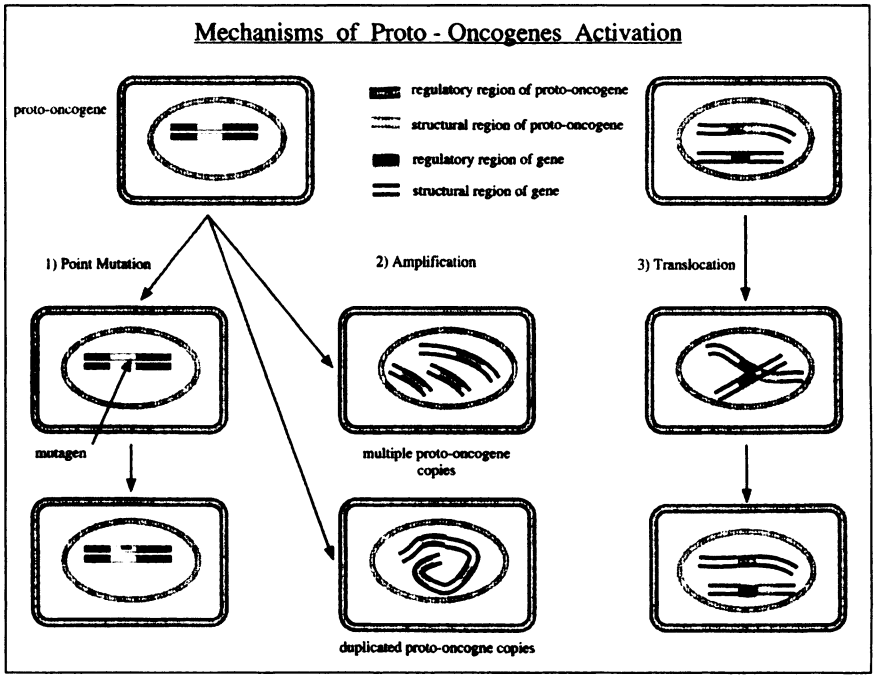

FIGURE 3. Activation of oncogenes. Structural and regulatory changes account for oncogene activation. Point mutation, amplification and translocation mechanisms can activate an oncogene and increase function and amount of oncoprotein synthesized.

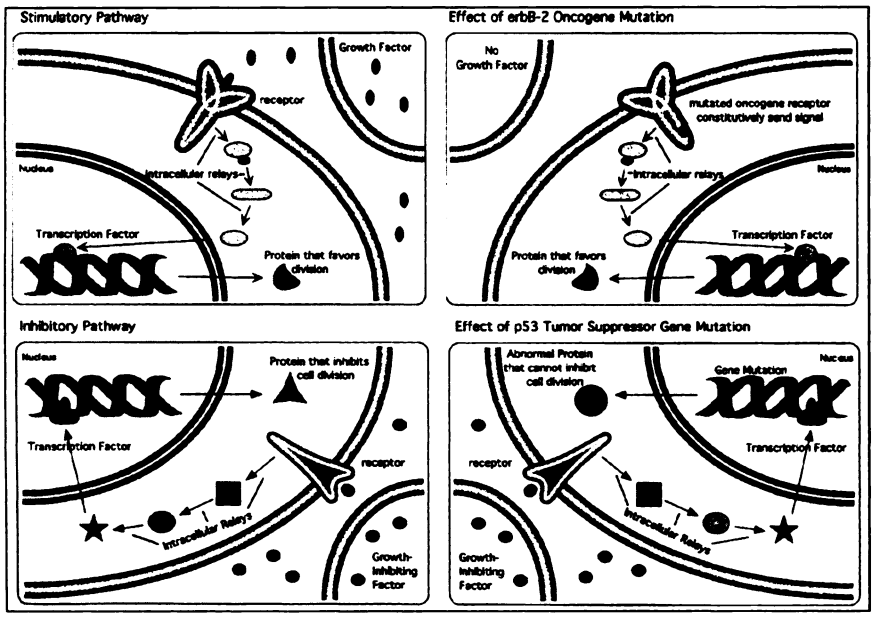

FIGURE 4. Effect of *erbB-2* and p53 mutation on stimulatory and inhibitory cell growth pathways. Mutated *erbB-2* is constitutively activated and sends permanent signal to intracellular protein cascade that ultimately leads to cell division. Mutation of p53 gene generates nonfunctional protein unable to bind to DNA target sequences and hence inhibits cell division.

and deletions account for the inactivation of tumor-suppressor genes. The best known example is the retinoblastoma gene. Retinoblastoma occurs either on a familial or sporadic basis. In the familial form, all children afflicted carry one intact and one defective copy of the retinoblastoma gene in all cells. On random, somatic inactivation of the other allele in a retinal cell, tumor growth is initiated. In sporadic cases, both copies of the gene are inactivated somatically in one retinal cell. In both instances, the absence of a functional pRb, either by complete absence (deletion) or mutation, results in the loss of the braking system on cell growth stimulatory signals.

The molecular basis underlying the carcinogenetic effect of p53 mutation is somewhat different. The p53 gene product is a monomeric polypeptide that binds to target DNA sequences as a tetramer. p53 mutations result in the production of a mutant polypeptide that generates a defective oligomeric complex (Fig. 4B). In the absence of a functional p53 protein, the *WAF1* gene is not transcribed, and the inactivation by the *WAF1* protein of the cyclins does not occur, resulting in the suppression of its growth-suppressive role. In addition, and since p53 controls negatively and indirectly its own transcription via the *MDM2* gene product, the amount of mutant p53 is usually increased in tumor cells.

TARGETING APPROACHES

The Imagene Concept

Over the past 5 y, investigators have been active in the development of antisense imaging technology. In 1994, Urbain et al. (*1*) showed that mouse plasmocytoma cells in vitro express high levels of IgA and preferentially retain phosphodiester and phosphorothioate antisense molecules of appropriate length that are complementary to the 5′ initiation codon region of the IgA mRNA. This retention was time dependent and proportional to the concentration of antisense in the milieu (*1*). The importance of the *erbB-2* oncogene in oncology is reflected by its overexpression in 25%–70% of breast cancer and its correlation with poor clinical and prognostic parameters such as tumor size, lymph node involvement, lack of hormone receptors and grade of anaplasia. Compared to normal breast cells, the overexpression of *erbB-2* results in a 1- to 128-fold increase in the *erbB-2* mRNA intracellular level (*2*). Using an 18-base antisense DNA sequence complementary to the 5′ region of the *erbB-2* mRNA adjacent to the initiation codon to label a human mammary cell line (MCF7), which expresses a low level of *erbB-2*, and a plasmocytoma cell line (MOPC315) as control, we observed a preferential, specific retention of *erbB-2* antisense molecules in the breast cancer cell line. We also showed that this retention is time dependent (*1*).

Scintigraphic in vivo imaging of an oncogene was first demonstrated in 1994 in a tumor-bearing mouse model. Using In-labeled antisense and a sense sequence aimed at the initiation region of c-*myc*, Dewanjee et al. (*3*) were able to obtain an image of a xenograph tumor implanted in the flank of a mouse. In contrast to the sense sequence, which did not reach a concentration of more than 1% of the injected dose in the tumor, about 10 % of the radiolabeled antisense probe localized in the xenograph within the first hour, achieving a tumor-to-background ratio of 15 over a 24-h period (*3*).

The requirements for the use of antisense molecules in cancer therapy are well defined and can serve as criteria for their use in imaging (*4*). For successful antisense imaging, target cells should have a sufficient amount of mRNA oncogene product and retain specifically and selectively the antisense probes. To satisfy these prerequisites and design a successful marker a few parameters must be met. First, the targeted gene needs to be overexpressed in the tumor.

Second, the targeted sequence in the mRNA must be accessible to form a duplex with the antisense molecule. That region cannot be involved practically in a hairpin structure or bind to a regulatory protein. Third, the RNA-antisense DNA duplex must be sufficiently resistant to the attack of ribonuclease H and have a melting temperature high enough to prevent dissociation of the base pairs at 37°C. In that respect and as a rule of thumb, the higher the guanine and cytosine (G-C) content of the duplex, the more stable the complex. This is explained by the three hydrogen bonds between the G-C bases versus the two bonds needed between adenine and thymine pairs. Last, but not least, the labeling of the antisense compound should not interfere with base pairing between the mRNA and antisense molecules.

Hybridization of radiolabeled DNA antisense with its intracellular mRNA target has yet to be demonstrated. Many important questions concerning synthesis, stability, cellular uptake-retention, intracellular specificity and toxicity of DNA oligonucleotides remain to be answered (*5–9*). Hnatowich (*10*) showed that both the phosphodiester and phosphorothioate ribose backbones seemed unsuitable for radiopharmaceutical applications, because of their unfavorable pharmacokinetic properties. Fortunately, DNA backbone derivatives are being actively developed, most by biotechnology companies (*11,12*). Because of their lower affinity to proteins, peptic nucleic acid oligomers seem to be more suited for radiopharmaceutical applications (*13*).

At this point, we can only hope that this innovative approach will mature rapidly to produce exquisitely specific radiopharmaceuticals for successfully detecting and imaging cancers that express specific oncogenes or tumor-suppressor genes.

The Oncogene Receptor-Ligand Approach

Cell membrane tumor-specific alterations can be used as potential targets to distinguish between tumors from growth-arrested normal tissues. For instance, tumor cells can harbor phenotype changes resulting from the reactivation of oncofetal genes, such as the α feto protein and carcinoembryogenic antigen (CEA) genes, or the overexpression of oncogenes, such as EGFRs. In some instances, malignant transformation results in the expression of other genes, such as somatostatin and the multiple drug resistant genes whose end product is suitable for targeting. The cytogenetic alterations present in these instances have been used advantageously and developed to detect, evaluate and treat neuroendocrine tumors bearing an increased amount of somatostatin receptors and tumors resistant to chemotherapeutic drugs. Excellent current reviews on these topics are available (*14,15*) and will not be discussed here because the genes affected are neither oncogenes nor tumor-suppressor genes.

Over the past 15 y, radioimmunoscintigraphy using monoclonal antibodies (Mabs) against membranous, tumor-specific proteins such as CEA and tumor associated glycoprotein 72 (TAG-72) have been used to detect and sometimes treat these tumors. Many clinical trials describing the results obtained with Mabs targeting various types of cancers have been published. Radiolabeled Mabs have proven to be valuable in the management of patients with adenocarcinoma of the colon, ovary and prostate (*16*). Mabs recognizing the cytoplasmic product of the ras proto-oncogene have also be used to image human breast cancer xenograft in animal models (*17*).

Murine Mabs against epitopes on the extracellular domain of the *erbB-2* receptor protein have been developed for detection, imaging and therapeutic purposes. Some have been able to localize breast tumor in tumor-bearing mice models (*18,19*) and in humans (*19*). In other instances, the antibodies have exerted remarkable and specific in vitro and in vivo inhibitory effects on growth in cells and tumors expressing *erbB-2* in animal models and, more recently, in phase Ia/Ib human trials (*20–25*). Despite the demonstration of tumoral immunodetection and immunotherapy, whole IgG molecules and their Fab fragments have not yielded consistent responses in the past. This can be attributed to the large size of the molecules that clear slowly from the circulation, distribute to normal organs and diffuse poorly from the vasculature to the tumor (*26,27*). In addition, the clinical use of these products has been seriously impaired by the heterogeneity of tumor antigen expression (*28*) and immunogenicity of murine antibodies (*29*).

Several approaches have recently been taken to overcome these limitations. First, recombinant Fv fragments have been engineered to target specifically and successfully epitopes on the extracellular domain of tumoral antigens, particularly *erbB-2* (*30–32*).

Second, by exploiting the direct efficacy of Mabs to downregulate *erbB-2* (*23,33–35*), it has also been demonstrated that small molecules derived from the complementarity-determining regions of these antibodies could be used to design organic mimetic peptides that mediate similar receptor binding and biological effects (*36*). This latter concept is especially attractive for imaging, because of the favorable pharmacokinetic and labeling characteristics of small peptides. Using a 13 amino acid-cyclized peptide derived from the humanized 4D5 Mab (*37*), which is highly specific for *erbB-2*, and confocal microscopy and flow cytometry, we recently have been able to successfully image and quantitate *erbB-2* expression on breast cancer cells (Fig. 5). Using the same approach and a synthetic decapeptide derived from a Mab against the pan-carcinoma cell surface antigen, polymorphic epithelial mucin, Sivolapenko et al. (*38*) were able to successfully detect and image small focal breast tumor and metastatic lesions.

Over the past decade, combinatorial libraries have revolutionized screening efforts for biologically active molecules. Another promising approach to identify peptide ligands to oncogene and tumor-suppressor gene receptors consists of the screening of peptide libraries displayed at the surface of filamentous phages (*39*). In this technique, a random phage display peptide library, in which a panel of peptides expressed in the coat of the virion, is screened on an

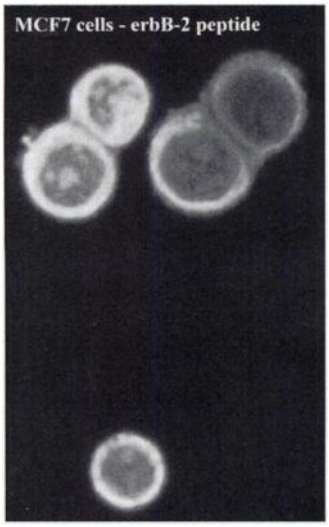

FIGURE 5. MCF7 cells tagged with fluorescently labeled peptide. This picture, obtained with confocal microscope, demonstrates labeling of membrane of MCF7 cells with cyclic peptide labeled with Oregon Green®. This 13 amino acid peptide was derived from CDR3 region of Mab specific for epitope of extracellular domain of *erbB-2* receptor.

immobilized target or against cells or organs to identify specific peptide sequences specific to an antigen, particular cells or selective organs (*40–43*). This approach has been used successfully to identify high-affinity antagonists to the human urokinase receptor and the fibronectin receptors that are key mediators in tumor cell invasion (*44,45*). The introduction of cysteine residues flanking the peptide sequence allows the formation of disulfide bounds and therefore cyclic peptide constructs with higher affinity and stability (*46*).

OTHER VENUES

Advances in solid phase peptide synthesis have dramatically broadened the flexibility and ease of designing specific peptides with branched homo or hetero sequences (*47*). These multimeric peptides demonstrate higher binding affinity than their monomeric counterparts and have been used successfully to inhibit protein tyrosine kinases, detect specific antibodies and induce cellular immune responses. A variation of branched peptides called loligomers, incorporating cytoplasmic translocation or nuclear localization signals, are rapidly internalized and accumulate in target compartments inside the cells (*48*).

This approach could be applied advantageously in radiochemistry to develop specific ligands to detect and image membranous, cytoplasmic or nuclear oncogenic target compounds.

Another class of very promising molecules with potential application in imaging are aptamers. Aptamers are nucleic acid molecules that bind to proteins and can be tailored to probe a specific target. DNA and RNA aptamer sequences that specifically bind dopamine, DNA polymerases, the hepatitis C virus NS3-protease and other regulatory proteins have been developed (*49–50*). In contrast to antisense molecules that complement their nucleic acid target (the sense molecule) in a strictly base pair-specific manner, aptamers bind to amino acid sequences within a specific protein. Aptamer libraries against various proteins are now being constructed using a combinatorial screening method (*51*). This elegant technique could be used to detect and image tumor cells producing an excess of oncogenic or nonfunctional tumor suppressor protein.

CONCLUSION

The discovery over the past three decades of the basic, fundamental, molecular mechanisms of carcinogenesis have unveiled numerous potential imaging targets. Antisense, aptamers, peptidomimetic and loligomeric probes are being actively developed to detect and image specific cells carrying activated oncogenes and tumor-suppressor genes that are responsible for cancer. At the dawn of the 21st century, the concept of imaging genes with molecular probes has become a virtual reality.

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