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Meeting Report

Fifteenth Annual Pezcoller Symposium: Molecular *in Vivo* Visualization of Cancer Cells

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Abstract

Recent advances in the understanding of the molecular phenomena underlying neoplastic development are leading to the recognition of metabolic features characteristic of the cancer cell. It has become possible to visualize the presence of these cells *in vivo* and to follow their progression toward increasing anaplastic behavior through measurements of molecular markers that can be achieved by physical or biochemical means.

Introduction

This symposium was focused on the molecular visualization of cancer cells and its potential clinical application in diagnosis, prognosis, selection of individualized therapies, and monitoring of tumor progression. Discussed were approaches using fluorescence or bioluminescence to identify cancer cells, advances in positron emission tomography (PET) and magnetic resonance (MR) for the imaging of tumors and their vascularization, the studies of proteomics and metabonomics as indices of tumor physiopathology, the possibility of imaging tumors by using small molecular probes, and the potential of these approaches for clinical applications.

Fluorescence and Spectral Imaging

Roger Tsien discussed visualization of gene products and small molecules and focused on genetically targetable indicators, of which the most recent class are chimeric substrates that alter intramolecular fluorescence resonance energy transfer and induce a change of color when phosphorylated by particular tyrosine or serine protein kinases. These indicators consist of cyan and yellow fluorescent proteins linked by a kinase substrate and phosphoaminoacid-binding domain. Indicators for PKA, Akt/PKB, PKC, EGFR, Src, and Abl have been or are being developed using this general strategy. Inhibition of phosphatases usually increases the extent of phosphorylation of the indicator. Fluorescence resonance energy transfer can also reveal dynamic protein-protein interactions in living cells, especially if one first suppresses the tendencies of fluorescent proteins to oligomerize. The most recent monomeric mutants have excitation and emission maxima as long as 605 and 632 nm, respectively. Short tetracysteine motifs have also been created, which can be labeled in live cells with membrane-permanent biarsenical dyes. Such labeling occurs with picomolar dissociation constants and represents a spectroscopically versatile, organic synthetic alternative to naturally fluorescent proteins. Unique applications include green *versus* red pulse-chase labeling of old *versus* new copies of the same protein, electron-microscopic localization, and chromophore-assisted light inactivation of a chosen protein, as was shown in studies of connexin trafficking, Golgi reorganization, and calcium channels. Chromophore-assisted light inactivation can be used to specifically inactivate the labeled protein *in situ* and constitutes a genetically targeted, microscopic version of photodynamic therapy.

As discussed by Richard Levenson, spectral imaging technology creates images with individual optical spectra at every pixel. In combination with appropriate mathematical tools, this capability can reveal complex and useful spectral information in common samples. Cambridge Research Instruments has developed two different ways of accomplishing spectral imaging, one based on liquid crystal tunable filters and one on a tunable light source. liquid crystal tunable filter-based devices transmit light in a number of wavelength ranges. The peak transmission can be electronically switched in under 100 ms. Such tunable filters are well suited to fluorescence-based analyses, *e.g.*, multicolor fluorescence *in situ* hybridization or green fluorescent proteins (GFPs) with overlapping emission spectra, and for the identification and elimination of interfering autofluorescence. They can simplify the approach to fluorescence resonance energy transfer-based assays. In addition, they can give information on spectra of fluorescent reagents *in situ* and can monitor spectroscopic shifts because of a dye's sensitivity to changing intracellular environments. Normally, the greenish-yellow autofluorescence generated by unlabeled mouse tissues can obscure the desired GFP signals. However, by using spectral imaging to generate GFP- and autofluorescence-spectra, the original spectral data set can be mathematically resolved into separate images. This procedure can enhance sensitivity. A novel imaging approach restricted to brightfield (nonfluorescence-based) microscopy replaces the standard halogen lamp at the rear of most microscopes and can deliver any combination or intensity of wavelengths from 420 to 700 nm, including white light. It is valuable for facilitating multiplexed immunohistochemistry procedures. Analysis of spectral images may require only spectral signatures and simple segmentation algorithms but may also reward the use of spectral and spatial analysis and advanced machine-learning techniques. Using either system, it was shown that conventional H&E- or Papanicolaou-stained pathology sections can contain sufficient spectral information to allow the classification of cells of different lineage or to separate normal from neoplastic cells.

Robert Hoffman described a dual-color fluorescence imaging model of tumor-host interaction based on a red fluorescent protein-expressing tumor growing in GFP-transgenic mice. This model enables visualization of the tumor-stroma interaction, including tumor angiogenesis and infiltration of lymphocytes in the tumor. All of the tissues from the transgenic line, with the exception of erythrocytes and hair, fluoresce green under blue excitation light. B16F0 mouse melanoma or breast carcinoma cells were transduced with the pLNCX2-DsRed-2-RFP plasmid. The B16F0-RFP tumor and GFP-expressing stroma could be clearly imaged simultaneously in excised tissue. Dual-color imaging enabled resolution of the tumor cells and the host tissues down to the single cell level. In SCID mice HT-1080 cells were transduced with either GFP or red fluorescent protein; 3×10^6 cells from each color clone were mixed and injected into the same footpad. Six weeks after the injection, the mice were sacrificed, and the colonies on the lungs were evaluated under fluorescence microscopy. Most colonies showed pure green or red fluorescence. However, if 1×10^6 cells from each clone were mixed and injected into the tail vein, almost all lung colonies showed mixed fluorescence. If 1×10^5 cells from each clone were mixed, lung metastasis were pure green or red fluorescence and thus were clonal. In collaboration with Dr. Scott Lowe, it was found that in lymphoid tumor cells labeled with GFP, cyclophosphamide was very effective in tumors with normal P53 and normal *Bcl*II content, partially effective in $P53^{-/-}$ tumors where tumor growth recurred, and ineffective in mice overexpressing *Bcl*II with normal P53. Using orthotopic tumor models labeled with GFP, tumor cells metastasized to organs, including liver, bone, lung, and brain could be visualized by whole-body imaging. Drug effects can be monitored in these models by whole-body imaging.

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Note: This symposium was held June 12–14, 2003, in Rovereto, Italy. The symposium was cochaired by Drs. Enrico Mihich (Roswell Park Cancer Institute, Buffalo, NY), William Kaelin (Dana-Farber Cancer Institute, Boston, Massachusetts), and Giulio Draetta (European Institute of Oncology, Milan, Italy).

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Advances in PET and MR

Zaver Bhujwalla discussed her development and application of noninvasive MR, imaging (I), and spectroscopy (S) techniques to understand the role of vascular, physiological, and metabolic properties in cancer invasion and metastasis. The MR studies were performed with human breast and prostate cancer cells maintained in culture or grown as solid tumors in immune suppressed mice. Cancer cells were grown on Matrigel to dynamically track invasion and simultaneously characterize oxygen tensions and physiological and metabolic parameters. A layer of endothelial cells between the Matrigel and cancer cells can be added to understand the impact of the presence of endothelial cells on cancer cell invasion during normoxia and in the presence of hypoxia and extracellular acidosis. Both invasion and vascularization were required for metastasis to occur. Significant differences were noted between metastatic and nonmetastatic human breast and prostate cancer models. The metastatic phenotype is characterized by high total choline, high lactate and low extracellular pH, and high permeability. Using a combined magnetic resonance imaging (MRI)/magnetic resonance spectroscopy (MRS) and optical imaging approach in studies of tumors derived from cancer cells engineered to express fluorescence under hypoxia, metabolic, extracellular pH, hypoxia, and vascular images from colocalized regions within a solid tumor were acquired and their dynamics during growth and following therapy evaluated. Combined MR and optical imaging studies demonstrated that regions of low vascular volume and high permeability were also hypoxic. The ability of MRI to detect the antiangiogenic effect of TNP-470 in a prostate cancer model was also discussed.

Ronald Blasberg outlined molecular-genetic reporter gene imaging and the use of combined PET and optical imaging technology, as were the advantages and limitations of these approaches. A reporter gene can be placed under the control of different promoter/enhancer elements. These elements regulate reporter gene expression and can be used to monitor endogenous cell processes and signaling as well as cell trafficking by noninvasive imaging. The transcriptional control of endogenous gene expression, posttranscriptional modulation of gene expression, and protein-protein interactions can be imaged. The coupling of reporter genes (*e.g.*, HSV1-tk and eGFP) provides the opportunity for combined PET and optical imaging, where a single bicistronic transcription cassette is used for coexpression of two genes that are linked in a fixed and definable manner. One segment of the fusion gene cDNA corresponds to the PET gene and another segment corresponds to the "optical" gene. In the case of TKeGFP, the functional imaging characteristics of both components (PET and optical) are retained. The TKGFP fusion allows for *in vitro* (or *in situ*) assays by fluorescence microscopy and fluorescence-activated cell sorting analysis, by whole body optical fluorescence imaging, and by noninvasive *in vivo* PET imaging with suitable tracers using a gamma camera, single-photon emission computed tomography, and PET. A close spatial correlation between the signals derived from both subunits of TKeGFP in a rat brain tumor xenograft model using fluorescence imaging and quantitative autoradiography was shown. The ability to correlate microscopic fluorescence imaging with quantitative autoradiography provides the opportunity to define spatial relationships between different reporter systems and to compare several different biological processes at the same time and at the level of $\sim$50–100-μm spatial resolution. A mutant thymidine kinase (HSV1-sr39tk)-Renilla luciferase fusion reporter construct (tk20rl) was recently developed for both PET and optical imaging. A good correlation was shown between the PET (microPET) and optical (charge-coupled device camera) readouts of the dual reporter system. Alternatively, a PET reporter and a bioluminescence reporter can be linked through an internal ribosomal entry site (IRES) to express two separate gene products. The IRES element enables translation initiation within the bicistronic mRNA, thus permitting gene coexpression by cap-dependent translation of the first cistron and cap-independent, IRES-mediated translation of the second cistron. However, fusion and IRES reporter constructs are not without problems. The fusion construct may result in a nonfunctional gene product. Modulation of the fusion mRNA or a change in the clearance of the fusion protein may also be sufficiently different from its unfused counterparts and could have a significant impact on the level of the fusion gene product. Fusion proteins are larger and are more likely to generate an immunological response. It remains to be demonstrated whether IRES-based vector expression will be a reliable indicator of transgene coexpression in different tissues.

Fluorescence and bioluminescence imaging of reporter-gene expression provides an alternative to more expensive imaging modalities such as PET and MR, and they provide real-time data of gene expression in small animals. However, *in vivo* optical imaging techniques are limited by depth of light penetration, photon scatter, and accuracy of measurement. The use of dual or triple modality reporter constructs (PET, fluorescence, and bioluminescence) overcomes many of the shortcomings of each modality alone.

Jerry Glickson outlined the development of novel contrast and photodynamic therapy agents for MR and optical imaging. A number of molecular imaging agents were developed; targeting of low-density lipoprotein receptors is being used for detection of tumors that overexpress these receptors. Imaging of low-density lipoprotein receptor by near IR optical imaging is achieved by using low-density lipoprotein labeled with tricarbocyanine-labeled cholesteroyl oleate as well as with pyropheophorbide and bacteriochlorophyll cholesteroyl oleate and also by near IR imaging of apoE-labeled liposomes containing indocyanine green. Labeling of LDL with gadolinium chelates for MRI detection has also been achieved. Delivery of near IR dyes and photodynamic therapy agents via GLUT1 and GLUT3 transporters has been achieved with 2-[2-deoxyglucose] adducts (2DG) of tricarbocyanines, pyropheophorbide, and bacteriochlorophyll. Uptake is inhibited by glucose, consistent with delivery via glucose transporters. In rats with s.c. 9L gliomas, low temperature fluorescence measurements confirm localization of these agents in tumor, liver, spleen, muscle, and kidney but not in brain. Selective oxidation of tumor mitochondria by the photodynamic therapy agent was seen. Imidazole adducts of 2DG have been prepared for measurement of intracellular pH of *in vivo* tumors.

Thomas Meade discussed chemical opportunities to increase contrast in identifying biological targets by MRI microscopy. Contrast agents are useful for identifying anatomical features, can be taken up by receptor-mediated means, targeted, and delivered into cells, and can be biochemically activated by expression of specific genes. To permit the direct observation of ongoing developmental events in living embryos, the descendants of individual precursors in an intact embryo were labeled by microinjection of a stable, nontoxic, membrane impermeable MRI lineage tracers. One could fully reconstruct the cell divisions and cell movements responsible for any particular descendant(s). A number of small molecule chaperones have been developed that include arginine and aromatic molecules. They are capable of transporting the agent inside a cell in relatively high yield without interfering with the activation of the agent by an enzyme or the binding of Ca^{2+}. Molecular MR probes that are biochemically activated *in vivo* have been investigated and reported in the form of an acquired three-dimensional-MR image. The modulation is triggered by the following: (*a*) enzymatic processing of the contrast agent; and (*b*) reversible binding of an intracellular messenger. Contrast agents that are activated *in vivo* by matrix metalloproteases, caspases, and kinases are being prepared.

Harvey Herschman discussed noninvasive imaging of gene expression by micro-PET under various conditions. Reliable systems were developed that permit investigators to monitor gene expression following somatic DNA transfer, cell transfer, and in transgenic animals. Reporter genes are used in which the protein products sequester positron-labeled reporter probes. The reporter gene-dependent retention of the positron probes in living animals is detected by PET. Two PET reporter gene (PRG) systems were developed, one based on enzyme-dependent sequestration of a positron-labeled reaction product, the second based on receptor-dependent sequestration of a positron-labeled ligand. Herpes virus simplex type I thymidine kinase (HSV1-tk) was used as a PRG and positron-labeled acycloguanosines as the PET reporter probe. A second generation HSV1-TK PET reporter gene/acycloguanosine PET reporter probe system was also developed that has a greater affinity for acycloguanosines and a reduced affinity for thymidine, increasing the efficacy of this PET reporter probe/PRG system. A side-chain labeled form of penciclovir was the best acycloguanosine PET reporter probe for the modified HSV1-sr39tk PET reporter gene. The dopamine D_2 receptor and positron-labeled spiperone, a D_2 receptor antagonist, was identified as a second PRG/PET reporter probe system. A second generation D_2 receptor PRG was developed that uncouples ligand binding from activation of signaling pathways initiated by dopamine occupancy of the D_2 receptor. *In vivo* it was shown that PET measurement of a PRG placed distal to an IRES accurately reflects expression of a second gene placed proximal to the IRES. Tumor-specific T lymphocytes taken from a tumor-bearing animal were transfected with a retrovirus expressing both the HSV1-tk PRG and GFP. Infected T cells were isolated by flow cytometry to enrich for the population expressing the PRG. These immune

cells were injected into tumor-bearing animals and then observed—by monitoring HSV1-tk dependent retention of an acycloguanosine PET reporter probe—to localize to antigen-positive but not antigen-negative tumors. In contrast, HSV1-TK-labeled, control lymphocyte populations did not accumulate at either tumor site. A positron-labeled acycloguanosine PRG was used to image expression of the *HSV1-tk* gene expressed from the murine albumin promoter in a transgenic mouse created by Eric Sangren. This mouse has a chimeric [albumin promoter][HSV1-tk] gene present in all cells but should only express the HSV1-TK protein in the liver. Micro-PET analysis of HSV1-TK levels, as determined by noninvasive imaging of retained hepatic PRG, demonstrated that transgenic PRG levels accurately reflect the level of endogenous albumin gene expression in this model system.

Genomics, Proteomics, and Metabolomics

John Lindon outlined new developments in studies concerning metabonomic approaches with particular reference to their transfer from the laboratory to the clinic. Metabolic changes are real-world end points, and metabonomics provide a useful connection between the otheromics platforms and pathology. Although mass spectrometry, gas chromatography-mass spectroscopy, high-performance liquid chromatography, and optical spectroscopic techniques can produce metabolic signatures of biomaterials, nuclear magnetic resonance (NMR) has the advantages of being nondestructive, applicable to intact biomaterials, and intrinsically more information rich with respect to molecular structure elucidation. NMR allows biochemical studies on intact tissues and cells using a technique known as magic-angle-spinning NMR, which preserves the samples for other studies. The application of NMR-based metabonomics in toxicological and clinical investigations has been demonstrated, and recently, its fundamental value in characterizing metabolic consequences of genetic variation in mammalian systems and in identification of metabotypes or metabolic phenotypes, which result from a combination of genetic and environmental factors, has been indicated. An important potential role for metabonomics is to direct the timing of proteomic and genomic analyses to maximize the probability of observing biological transitions that predict functional outcomes. High-frequency ^{1}H NMR spectroscopy is particularly useful in biochemical investigations in that it is sensitive (low ng detection limits are possible), and nearly all metabolic intermediates have unique ^{1}H NMR signatures. Metabolic profiles of biofluids reflect both normal variation and the pathophysiological impact of toxicity or disease on single or multiple organ systems. Even a one-dimensional high frequency ^{1}H NMR spectra of urine typically contains many thousands of sharp lines from a myriad of mainly low molecular weight compounds. Plasma contains low and high molecular weight components. Directly coupled chromatography-NMR spectroscopy methods can be used, especially for drug metabolites, the most powerful of these hyphenated approaches being HPLC-NMR-MS. Biofluid NMR is useful for rapid screening especially when performed via flow injection methods. However, direct target organ toxicity or disease fingerprints can also be investigated by using (magic-angle-spinning) NMR of intact tissues. Preliminary analysis of the data usually involves the application of unsupervised pattern recognition methods such as principal components analysis or cluster algorithms that assume no prior knowledge of sample class. Specific pathologies can be characterized by principal components analysis trajectories where clustering of sample coordinates reflects an intrinsic similarity in biochemical composition and allows differentiation between classes. Information relating to biomarkers can be extracted from the analysis. It can be demonstrated that metabonomics information is obtainable on a wide range of applications as effects of aging, identification of inborn errors of metabolism, early development of atherosclerosis, presence of osteoarthritis *versus* osteoporosis, coronary diseases involving 1, 2, or 3 vessels, or the presence of parasitic diseases in urine from Ivory Coast individuals. Metabonomics can also be used for phenotyping mutant or transgenic animals and the investigation of the consequences of transgenesis; the transfection process itself can cause significant metabolic differences. Metabonomics can give insight into the metabolic similarities or differences between mutant or transgenic animals and the human disease processes that they are actually intended to simulate.

Michael Stratton discussed his systematic genome-wide searches to find new cancer genes. Since the advent of recombinant DNA technology, a major aim of cancer research has been the identification of genes that are mutated and causally implicated in oncogenesis (cancer genes). To date, the list currently compiled includes ~300 cancer genes (including genes that are mutated in the germ-line and predispose to cancer and genes that are somatically mutated), so 1% of the genes in the genome already are known to contribute to oncogenesis. Approximately 150 genes are somatically mutated in leukemias, lymphomas, and sarcomas, whereas ~80 genes are mutated in all of the other types of cancer. To identify the remaining cancer genes, the human genome sequence will be a template subserving genome-wide searches. A full description of changes at the DNA level in cancer cells will require information on all types of abnormalities: copy number changes; rearrangements; point mutations; and methylation. It is currently necessary to develop platforms that detect each of these kinds of change separately. Using the whole genome sequence, systematic genome-wide searches for point mutations (base substitutions and small insertions/deletions), copy number changes, and rearrangements have been started.

An early-stage of a systematic search for point mutations in the human genome revealed somatic mutations in the *BRAF* gene in human cancer. BRAF is a member of a family of three serine/threonine kinases that also includes RAF-1 (also known as CRAF) and ARAF. RAF proteins are recruited to the cell membrane and activated by RAS proteins in the RAS-RAF-MEK-ERK-MAP kinase signal transduction pathway. Mutations of *BRAF* have been found in 70% of melanomas and in a lower proportion of papillary thyroid cancer, colorectal cancer, borderline ovarian tumors, and bile duct adenocarcinomas. In colorectal cancers, *BRAF* mutations occur at a similar stage of colorectal oncogenesis as *RAS* mutations. *BRAF* mutations are located within the kinase domain: >80% of the mutations cause a single amino acid change (V599E) within the activation segment, whereas the remainder are accounted for by a diverse amino acid substitutions within the activation loop or G loop (ATP binding region) of the kinase domain. The common *BRAF* mutation (V599E) does not coexist with *RAS* mutations, whereas the less common mutations often do coexist. Using anti-RAS antibodies, it was shown that cancer cell lines with V599E grow independently of RAS signaling, whereas those with the less common *BRAF* mutations are still dependent on RAS signaling. *BRAF* mutations usually cause activation of the BRAF kinase activity and are transforming in NIH3T3 cells. The patterns of *BRAF* mutation and their function generated a plausible new target for drug development.

Systematic genome-wide mutational screens also reveal insights into global patterns of mutation that differ between individual cancers and cancer types and can provide information on the history of the cancer, including past exposures or DNA repair defects. Ultimately, these searches are expected to provide information on core parameters of human cancers.

Jan van der Greef outlined the use of system biology to discover biomarkers in human diseases included cancer. In modern drug discovery, the primary source for target identification and validation comes from genomics research and is followed by advanced proteomic approaches. Later in the discovery and development process, biomarkers/surrogate markers become necessary. The concept of system biology is the integration of the information at the different molecular levels of living organisms: transcriptomics; proteomics; and metabolomics. Bioinformatics is a key tool in this process that enables the integration of data on pathways involved in disease progression and also permits the study of perturbations of a system using a drug. The focus is on biomarker fingerprints. This was done in multiple sclerosis patients and for 20 other neurological diseases. A strategy for biomarker discovery based on systems biology was described by highlighting the concept of systems biology and biomarker discovery from a healthy/disease perspective and the impact on the drug discovery/drug development; body fluid profiling techniques based on a novel proteomics and metabolomics platform; a BioSystematics approach, including data handling, based on sophisticated pattern recognition techniques. Proteomic measurements can be done by a sophisticated multidimensional liquid chromatography (LC)/mass spectrometry platform in combination with pattern recognition tools as was highlighted by a cancer biomarker study showing clustering of the fingerprints based on gender, ethnic background, but most importantly on characteristic serum biomarker profiles.

Bioluminescence

Anthony Campbell described the use of imaging of genetically engineered Rainbow proteins. Bioluminescence is the emission of visible light from living organisms. It occurs in a wide range of terrestrial and marine organisms from some 17 different phyla. Bioluminescence requires a small organic molecule,

the luciferin, oxygen or one of its metabolites, and a protein catalyst, the luciferase. Some systems require cofactors such as NAD(P)H, FMN, ATP, or ions such as calcium. Genetic engineering of the DNA from a variety of bioluminescent organisms provides probes for free Ca^{2+}, ATP, oxygen metabolites, and gene control. Chemical events can be measured and imaged in defined compartments of living cells. Using a fiber optic, it is possible to image the light emission from individual cancer cells without the need for a microscope. Rainbow proteins were engineered so that they change color and/or intensity when they react with a substance of biomedical interest. The three types of Rainbow protein are as follows: firefly luciferase linked to aequorin, which can measure ATP by yellow light and Ca^{2+} in the nuclear or cytosolic membranes by blue light; a covalent modification recognition site engineered into the luciferase; and a linker engineered between an energy transfer donor-acceptor pair and affects the width of the band. Thus, indicators have been developed for protein kinases and proteases that work inside living cells. A photon counting camera suitable for detecting bioluminescence has been adapted to image four colors simultaneously, and calibration of the spectra was done.

The energy transfer Rainbow proteins are a generic, ratiometric indicator system for proteases. The flash spectrum showed that there was a complete shift from aequorin to that of GFP, which was reversed when caspase cleaved the linker. Caspase activation was measured before apoptosis. Bioluminescent indicators were used to uncover a signaling system within the endoplasmic reticulum that communicates with other parts of the cell. This signaling system determines whether a cell fires a global Ca^{2+} signal, generates Ca^{2+} oscillations, defends itself against stress, or dies via apoptosis. The two key signaling proteins within the endoplasmic reticulum seem to be calreticulin and Bi-P(grp78). Discrepancies between bioluminescent and fluorescent measurements were found.

As Tullio Pozzan indicated, Ca^{2+} is a universal second messenger that modulates a number of highly different biological functions from fertilization to secretion, proliferation, differentiation, and cell death. One of the key issues in determining the role of Ca^{2+} in physiological or pathological conditions is the availability of methods that allow the measurement of this ion not only in living cells but also at the subcellular level and under conditions as close as possible to those *in vivo*. Approaches were briefly described, which had been developed in Pozzan's laboratory to monitor Ca^{2+} at the subcellular level, and the applications of these methodologies to the study of apoptosis were discussed. Two types of approaches were used to study Ca^{2+} at the subcellular level: on the one hand, the use of classical fluorescent Ca^{2+} indicators and single or two photon confocal microscopy and, on the other, the Ca^{2+}-sensitive chemiluminescent or fluorescent proteins engineered to be targeted to different subcellular locations. It was shown how one can get information on Ca^{2+} handling in isolated cells, tissues (brain slices), or even *in vivo* (skeletal muscle fibers in the live anesthetized mouse). Data concerned with the role of Ca^{2+} in controlling apoptosis were also discussed. By different approaches (use of selective drugs, manipulation of extra or intracellular Ca^{2+} levels, overexpression of antiapoptotic proteins such as Bcl-2 or knockout of proapoptotic genes such as Bak and Bax), it was demonstrated that the level of Ca^{2+} in the endoplasmic reticulum is a key component in the pathway, leading to activation of apoptosis with stimuli such as ceramide, H_2O_2, or arachidonic acid. On the contrary, other apoptotic stimuli, in particular, those using the so-called BH3 pathway, appear to be totally insensitive to Ca^{2+} levels.

Christopher Contag discussed the *in vivo* use of bioluminescent reporters as a means of measuring gene expression, assessing tumor burden, and tracking tumor targets and immune effector cells. This is a sensitive *in vivo* cellular and molecular imaging tool that has a broad dynamic range and provides whole body images of living animals expressing optical tags. In oncology, the sensitivity of detection permits the study of minimal disease, either early in the disease course or minimal residual disease after therapeutic intervention, and enables the detection of micrometastatic lesions. Using multifunctional reporter genes, which encode both bioluminescent and fluorescent proteins, the *in vivo* trafficking studies are linked to the *ex vivo* assays that use fluorescence. To address the problem of minimal residual disease, cell therapies that use a cytotoxic population of cells with a natural killer T-cell phenotype were investigated. *In vivo* imaging of tumor burden revealed that the cell therapies were efficacious against lymphoma targets and, moreover, that these cells could be effectively redirected using bi-specific antibodies to tumor targets that lack the intrinsic expression of ligands for natural killer T-cell recognition. As dendritic cells are being investigated as mediators of antitumor immunity and as the basis of experimental vaccine strategies, the trafficking patterns of these cells were investigated in animals. Early disappearance of dendritic cells was apparent; however, signals from the surviving population could be followed for >100 days. Real-time observations of trafficking and proliferation of immune and tumor cells in intact animals showed the kinetics of these interactions. Imaging reporter gene expression in living animals provides critical spatiotemporal information about changes in cell growth, cell trafficking, and gene expression during normal and disease processes in the context of whole biological systems.

Clinical Application

Joseph Bertino discussed imaging of dihydrofolate reductase (DHFR) fusion gene expression. A novel method was developed to modulate gene expression *in vivo* by using exogenous DHFR fusion genes and systemic antifolate administration. Proteins of interest, *e.g.*, therapeutic transgene products such as HSV-TK are fused and thereby adopt DHFR regulation mechanisms, including translational up-regulation in response to antifolates such as trimetrexate. DHFR protein binds to its cognate RNA and prevents its own translation. Addition of antifolates prevents the binding of the DHFR protein to its own mRNA, thereby relieving translational autoregulation, leading to resumption of DHFR synthesis. An increase in intracellular levels of the fusion protein DHFR-HSV1 TK may lead to greater retention of the isotopically labeled substrate leading to greater image intensity as seen by PET scans or gamma camera imaging. A potential application of this technique is to detect in deep tissue noninvasively a specific molecular response following systemic drug treatment. Using human colon adenocarcinoma cells derived from a patient's liver lesion, a nude rat xenograft model for colorectal cancer metastatic to the liver was developed. Expression of a DHFR-HSV1 TK in the hepatic tumors was monitored in individual animals using the tracer [^{124}I]2′-fluoro-2′-deoxy-5-iodouracil-β-D-arabinofuranoside and a small animal microPET, whereas groups of rats were imaged using the tracer [^{131}I]2′-fluoro-2′-deoxy-5-iodouracil-β-D-arabinofuranoside and a clinical gamma camera. Growth of the human cancer cells in the rat liver was detected using MRI and confirmed by surgical inspection. In rats bearing hepatic tumors, which were retrovirally transduced to express the DHFR-HSV1 TK transgene, the fusion protein expression in the hepatic tumor of living rats was imaged using the tracer [^{124}I]2′-fluoro-2′-deoxy-5-iodouracil-β-D-arabinofuranoside and a microPET. Specificity of the signals was ascertained. Another study used the tracer [^{131}I]2′-fluoro-2′-deoxy-5-iodouracil-β-D-arabinofuranoside and a gamma camera to monitor two groups of transduced hepatic tumor-bearing rats. Before imaging, one group was treated with trimetrexate. Increased signal and tracer accumulation were seen. Thus, it is feasible to monitor DHFR-HSVTK fusion protein expression in hepatic colorectal tumor tissue in living animals.

Annick D. Van den Abbeele outlined clinical applications of molecular imaging with the example of imatinib mesylate (Gleevec) for the treatment of advanced gastrointestinal stromal tumors. The downstream effect of pathway-specific drugs does not necessarily lead to significant morphological changes. Bi-dimensional changes in tumor size on computed tomography or MRI may, therefore, not adequately reflect therapeutic response in the early phase of therapy. PET is well suited to evaluate the phenotypic characteristics of cancer cells and the effect of molecular-targeted therapy *in vivo*. The radionuclides used in PET imaging include, among others, carbon-11, nitrogen-13, oxygen-15, and fluorine-18. These can be incorporated into numerous compounds, including 2-deoxy-D-glucose, water, thymidine, methionine, choline, nitromisonidazole, and dopamine. The ability of PET to detect and quantitate picomolar concentrations of tracers within tissues results in a higher sensitivity relative to other imaging modalities, including MRI. The role of PET with the glucose analogue F-18-fluoro-2-deoxy-D-glucose (FDG) is currently being tested in patients with various cancers treated with new pathway-specific drugs. The role of FDG-PET as a surrogate marker of response to Gleevec therapy in patients with advanced gastrointestinal stromal tumors was presented. Objective response to therapy by computed tomography, time-to-treatment failure, and overall survival was used as the endpoints. A secondary objective was to determine the association between changes observed on FDG-PET and mutation status. The data confirmed the role of FDG-PET as a suitable surrogate marker of response to this drug. Preliminary results of the

effect of a multitargeted receptor tyrosine kinase inhibitor (SU11248) in patients with primary resistance to Gleevec were also presented and demonstrated the ability of FDG-PET to follow the metabolic response to this drug as a function of the therapeutic regimen.

Andrew von Eschenbach concluded the symposium and emphasized the fact that through the contributions of thousands of scientists in the cancer research community, we have reached the opportunity to make exponential progress. This inflection point is a consequence of the growth and intersection of a number of factors that includes increased understanding of cancer and a rapidly evolving portfolio of enabling technologies such as functional imaging. By using an integrative approach to the biology of cancer, it is now possible to aim for eliminating the suffering and death from cancer. . . hopefully by 2015.

Functional imaging will play a major role in this new era of molecular medicine because it is quickly becoming a critical tool in the lab and the clinic. At the bench, molecular imaging holds the promise of rapidly accelerating our understanding of the functional expression of the genome, proteome, and metabolome. In the clinic, functional imaging using many powerful new imaging agents coupled with PET, MRI, and ultrasound will enable real-time definition of pathways and evaluation of the effectiveness of new interventions. Armed with the ability of functional imaging to define tumor biochemistry and inform the design of appropriate therapeutics, the challenge goal to eliminate the death and suffering from cancer by 2015 will progress from a vision to a reality.

Appendix

The program committee consisted of the cochairs, David Livingston (Dana-Farber Cancer Institute, Boston, MA) and Lucio Luzzatto (Istituto Nazionale Ricerca sul Cancro, Genova, Italy). In addition to the program committee members, invited participants included: Joseph Bertino (The Cancer Institute of New Jersey, New Brunswick, NJ), Zaver Bhujwalla (Johns Hopkins University School of Medicine, Baltimore, MD), Ronald Blasberg (Memorial Sloan Kettering Cancer Center, New York, NY), Anthony Campbell (University of Wales College of Medicine, Cardiff, United Kingdom), Christopher Contag (Stanford University, Stanford, CA), Juri Gelovani (Memorial Sloan Kettering Cancer Center, New York, NY), Jerry Glickson (University of Pennsylvania, Philadelphia, PA), Hans Grunicke (Institute Med Chemie & Biochemie, Innsbruck, Austria), Harvey Herschman (UCLA Jonsson Comprehensive Cancer Center, Los Angeles, CA), Robert Hoffman (AntiCancer, Inc, San Diego, CA), Richard Levenson (Cambridge Research & Instrumentation, Inc, Woburn, MA), John Lindon (Imperial College, London, United Kingdom), Richard Mazurchuk (Roswell Park Cancer Institute, Buffalo, NY), Thomas Meade (Northwestern University, Evanston, IL), Marco Pierotti (Istituto Nazionale Tumori, Milan, Italy), Tullio Pozzan (Unviersity of Padova, Padova, Italy), Michael Stratton (The Wellcome Trust Sanger Institute, Cambridge, United Kingdom), Roger Tsien (University of California at San Diego, La Jolla, CA), Annick van den Abbeele (Dana-Farber Cancer Institute, Boston, MA), Jan van der Greef (TNO Pharma, Zeist, the Netherlands), Andrew von Eschenbach (NIH, Bethesda, MD).

The posters were presented by the following. Marian Terez (Center of Nuclear Medicine, University of Debrecen, Hungary); Irmgard Irminger-Finger (University of Geneva, Switzerland); Nadia Zakhary (National Cancer Institute, Cairo University, Egypt); Frederik Tack (Erasmus University of Rotterdam, the Netherlands); Nicole Schmitz (University of Bern, Switzerland); Christian Schnell (Novartis Pharma AG, Basel, Switzerland); Peter Allegrini (Novartis Institutes for Biomedical Research, Basel, Switzerland); Egidio Iorio (Istituto Superiore di Sanita, Rome, Italy); Dmitri Artemov (Johns Hopkins University, Baltimore, MD); Richard Mazurchuk (Roswell Park Cancer Institute, Buffalo, NY).