Meeting Report

Ninth Annual Pezcoller Symposium: The Biology of Tumors 1

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The Ninth annual Pezcoller Symposium was held in Rovereto, Italy, on June 4 to 7, 1997, and focused on molecular mechanisms underlying the biology of tumors. The genetic mechanisms underlying heterogeneity of tumor cell populations and tumor cell differentiation were discussed. Tumor cells closely interact with host cells and cell products having different functions, and these interactions play an important role in the biology of tumors. Interactions between tumor cells and cells of host defenses were discussed, with particular emphasis on the molecular basis of tumor recognition by the immune system. Interactions between cells were also discussed with reference to molecular mechanisms of cell regulation that are affected by or implemented through these interactions. The mechanisms of tumor vascularization were also discussed: without suitable angiogenesis, the tumor cannot grow and metastasize. Angiogenesis also provides a potential site of therapeutic intervention, and this makes it even more important to understand the mechanisms underlying it. Cell regulation and cell interactions are determined by activated genes through the appropriate and timely mediation of gene products. It is becoming increasingly important to develop methodologies that would allow us to measure differentially genes and gene products and thus validate many of the mechanisms of control proposed presently.

Genetic Determinants of Tumor Pathogenesis

DePinho discussed the role of INK4a in the pathogenesis of malignant melanoma. The 9p21 deletion/mutations are most frequent in both familial and sporadic melanoma. The 9p21 locus encodes at least three growth inhibitors, i.e., the ink4a- and ink4b-encoded p16ink4a and p16ink4b, respectively; ink4a also encodes p19ARF2. The difficulty in understanding this complex genomic organization is compounded by the frequent occurrence of large homozygous 9p21 codeletions. The ink4a gene was found to be the principal target in melanoma.

The causal role of the changes for the RTK-RAS-MAPK pathway consequent to RAS mutations in melanoma was unclear. Using ink4a knockout mice, cell culture transformation assays and tyrosinase-RAS transgenic mice, it was shown that ink4a is a bona fide tumor suppressor in vivo, and that in ink4a-deficient background, the RTK-RAS-MAPK pathway is etiologically important. However, the long latency and the absence of a metastatic phenotype in the tumors generated pointed to a weak oncogenic role of H-RASG12V under these conditions. The potent p19ARF, which is partially p53 dependent and suppresses transformation in vitro, supports an antioncogenic role for p19ARF; consistently, in vivo tyrosinase-RAS tumors show loss of heterozygosity with wild-type ink4a allele deletion including both p16INK4a and p19ARF regions, whereas wild-type p53 is retained.

As Berns pointed out, retroviral insertional mutagenesis is a useful approach to identify genes conferring selective phenotypes in as much as the retroviruses also leave a sequence tag at the DNA sites affected, thus permitting ready gene identification. Proto-oncogenes are activated by proviral insertion in lymphoid tumors. The study of insertional mutagenesis can be extended using mice with oncogenes in the germ line; infection of these mice preferentially leads to tumors carrying provirally induced changes synergizing with oncogenes in the transgenes. Transplantation of primary tumors in syngeneic hosts then allows outgrowth of more malignant clones characterized by genes likely to contribute to tumor progression or metastasis potential. Proviral tagging in mice with activated or disrupted proto-oncogenes can also be useful to search for genes acting in defined signal transduction pathways (complementary tagging). Studies using the Moloney murine leukemia virus were discussed using the approaches just mentioned. Retrovirus insertional mutagenesis was utilized: (a) to identify sets of oncogenes that synergize in the induction of lymphomas and to assign each of them to a complementary group in transformation (the activation of c-myc in Pim 1 transgenic mice was one of the examples discussed); (b) to identify genes involved in later stages of tumor progression, e.g., Fst1 and Tic1; and (c) to study the mode of action of these genes by identifying, through complementary tagging, the cellular pathways in which they act. The application of proviral insertional mutagenesis in transgenic mice could be manipulated and combined with other specific selection processes. Thus, following neonatal infection with Moloney murine leukemia virus, lifelong virema was established, which allowed sequential activation of genes contributing to tumor progression. The various tagging strategies permitted the study in mice of biochemical problems by a genetic approach analogous to the techniques pioneered in invertebrate model organisms.

As discussed by White, genetic markers can be used to localize human disease genes. After the first multiallelic locus PAW101 was described, thousands of human genetic markers, based on DNA sequence variation, have been characterized and ordered into chromosomal somats toward mapping new disease genes. Precise mapping is the final step of gene identification; the disease-related genes are
characterized by specific mutations associated with the evidence of the disease. The APC\textsuperscript{3} gene, the etiological agent in familial polyposis, was discussed as an example. This gene is frequently mutated also in sporadic polyps and in colon cancer and presents base pairs changes or deletions leading to prematurely terminated peptides. The APC protein immunoprecipitates jointly with β-catenin, the latter known to bind to E-cadherin and to mediate its link to the actin of cytoskeleton. APC was found to stimulate proteolytic release of free β-catenin protein. APC is located in the cytoplasm with foci at the leading edge of migrating cells and in the nucleolus; these locations are compatible with the APC protein being associated with the intermediate filament network. The overall results suggest a role of APC in regulating β-catenin levels. The β catenin/Tcf transcription complex induces several transcripts, among which is the prostaglandin synthetase enzyme COX-2, which is at high levels in most adenomas and is the target of nonsteroidal anti-inflammatory drugs like aspirin. It was concluded that colon cancer initiation may occur through a loss of APC function, leading to increased β-catenin-dependent induction of COX-2 and consequent increased prostaglandin levels.

As Gray pointed out, by using CGH, it is possible to identify regions of increased or decreased DNA copy numbers, with respect to normal controls, in target metaphase chromosomes of tumor. For example, in comparing two clinically similar breast tumors by CGH, many regions of increased and decreased relative copy numbers were found with numerous individual differences in profile of changes. This diversity in genetic signature is consistent with diversity in tumor progression and response to treatments. However, abnormalities may also be recurrent in different tumors and may identify specific patterns of cancer progression as found by CGH in breast and ovarian cancers. Studies examining, by fluorescence in situ hybridization, a region of increased copy numbers at 20q13 in breast cancer and at 3q26 in ovarian cancer were summarized. After the identification of a recurrent amplification at 20q13 that correlated with a highly malignant phenotype, two unique sequences were found, ZABC1 and AIBC1, which were consistently amplified and overexpressed at that site. The function of these new genes is being studied. Increased copy number at 3q26 were found in cancer of the ovary, lung, head and neck, and cervix. It was demonstrated that PIK3CA, the \( \text{M}_2 \), 110,000 catalytic subunit of PI3 kinase was located at that site; p110α is encoded by PIK 3CA and binds to activated tyrosine kinase receptors to initiate PI3-kinase signaling. It was found that p110α was overexpressed in ovarian cancer and was associated with increased PI3 kinase activity and with increased apoptosis after PI3 kinase inhibition. Genomic analysis of this type should provide a picture of the specific genetic events that are involved in the initiation and progression of specific human cancers.

Hanahan discussed lymphocyte-tumor interactions investigated in transgenic mice expressing SV40 large Tag under the control of the RIP Tag expression in pancreatic β cells, which leads to focal hyperplasia, tumor angiogenesis, and formation of solid tumors. In RIP1-Tag5 transgenic mice, preneoplastic lesions were infiltrated by CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and B cells, but despite this response, lethal, highly vascularized tumors develop that are not infiltrated by lymphocytes and kill the mice at about 30 weeks. In these mice, tumor antigen recognition processes were adequate, and no evidence of tumor-induced Fas-mediated immune suppression could be found. Evidence was obtained that HEVs, which mediate lymphocyte adherence and transendothelial migration, were low in noninfiltrated tumor areas. In double transgenic mice coexpressing Tag and a rearranged Tag-specific TCR, still no tumor infiltration of lymphocytes was noted. In RPIP1-Tag mice cross-bred with human B7-1 transgenics, tumors again had no infiltrating lymphocytes. On the basis of the observations as a whole, it was proposed that the tumor vessel endothelium is refractory to lymphocyte extravasation, and it was suggested that antiangiogenesis and T-cell regimens be combined for an effective treatment of cancer.

A set of high-density DNA arrays with complementarity to more than 6500 human expressed sequence tags was discussed by Gingeras. Using them, normal and breast cancer-specific gene expression profiles were obtained. Of all those observed, 85% were in the range of 1–50 copies/cell. More than 300 genes were expressed differently in normal versus transformed breast cells. Higher mRNA levels were observed in tumor for Heat2 neu oncogene and genes involved in its signal transduction pathway such as Grb-7, Raf, Raf, Mek, and ERK. Patterns indicating loss of wild-type p53 function were also seen; by using a DNA sequencing array, inactivating mutations in the p53 DNA binding domain and loss of heterozygosity were demonstrated. The methodologies used in these studies were described in detail.

The present status of the cDNA microarray technology was discussed by Melzer with reference to its potential applications toward providing large-scale analysis of gene expression. The essential aspects of microarray technology include the availability of a microarray robotic printer, an array scanner and image analysis software, and the availability of appropriate fluorescent probes; array elements must be selected to reduce the redundancy present in the expressed sequence tag database to obtain unique clusters. To facilitate data interpretation, it is necessary to use systems that allow rapid retrieval of information on each gene represented on a microarray. The microarray technology has been used in studies of tumorigenic melanoma cell lines and of a related chromosome 6 suppressed subline, as well as in studies of heat shock and phosphorl-ester-regulated gene expression in human T cells. In these studies, only a subset of the genes arrayed were altered; this implies that smaller arrays may have to be created that would be directed at answering specific biological questions. Microarray technology based on cDNA hybridization was critically compared with oligonucleotide array hybridization, and it was concluded that most likely both oligonucleotide and cDNA array technologies will coexist while the best applications for each of them are being identified.

Using a novel genome-wide sampling procedure called inter-(simple sequence repeat) PCR, Anderson estimated the minimum un互利 genomic events that had occurred in colorectal cancer cells. The evaluation was limited to tumor-specific bands not present in normal tissues; of the 171 bands found to be altered in 6 tumors, one-tenth to one-fifth had no normal tissue counterpart. Evidence of genomic instability was also found in seven adenomatous polyps, suggesting an early onset of genomic instability in colorectal disease.

A new protein-protein interaction domain was originally identified as a motif present in three copies at the NH\textsubscript{2}-terminus of the tyrosine kinase substrate eps15 and was named EH. As discussed by DiFiore, EH is conserved during evolution, and EH-containing proteins have heterogeneous characteristics and functions. The structures of Eps15 and of the closely related protein Eps15R were illustrated. The function of eps15 and eps15R are still unknown, but their roles in cell proliferation were suggested based on the finding that overexpression of eps15 can transform NIH 3T3 cells, and that the eps15 gene is rearranged with the HRX/ALL1 locus in acute leukemia translocations; moreover, eps15 and eps15R are associated in vivo with the clathrin

\textsuperscript{3} The abbreviations used are: APC, adenomatous polyposis coli; CGH, comparative genomic hybridization; Tag, T antigen; RIP, rat insulin gene promoter; HEV, high endothelial venule; TCR, T-cell receptor; E1V, Epi51 transmembrane; cdk, cyclin-dependent kinase; ECM, extracellular matrix; ER, estrogen receptor; PCD, programmed cell death; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; TAA, tumor-associated antigen; ICAM, intercellular adhesion molecule; NK, natural killer; STAT, signal transducers and activators of transcription; VEGF, vascular endothelial growth factor; PI3 kinase, phosphoinositol 3'-kinase.

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adapter protein complex AP-2. The discovery of the EH protein-protein interactions led to the identification of novel interactions in which eps15 and eps15R are involved. In addition to the relationships to growth indicated above, a regulatory role of EH-containing proteins in endocytosis was suggested by many of the results discussed, although the precise mechanism involved was not known; it is possible that EH-containing proteins function as adapter molecules, a hypothesis supported by the molecular structure of EH proteins and their capacity to oligomerize. Also, it was suggested that EH-containing proteins may represent centers of organization of cellular proteins that regulate protein and/or organelle sorting and transport.

**Genetic Determinants of Cell Cycle and Apoptosis**

Michalides discussed the deregulation of cyclin D, which occurs in cancer cells, and the consequences of this deregulation; changes in the expression of genes of cyclins and cdks controlling the cell cycle have a critical role in transformation and tumor progression. An increase of cyclin D after mitogenic stimulation leads to binding and activation of cdk4 and subsequent phosphorylation of pRb; this provides for the release of the transcription factor E2F, followed by the cascade of phenomena underlying progression through G1-S. The interrelationships between activation and inhibition of different cyclin and related kinases and the role of cell adhesion to ECM components in the control mechanisms determining these interrelationships were discussed; cyclin D1-cdk4 was indicated as a “sensor” of cells for growth and, together with cyclin E-cdk2, as “sensors” for attachment to ECM. In tumor cells, the coordinated control of the cell cycle by growth factors and the ECM, which is required for ordinate progression, is deranged. In fact, in most tumors, genetic alterations occur that alter the effects; overactivation of cyclin D1 in transgenic mice depend on growth factors and on cell adhesion for proliferation. Estradiol-induced proliferation seems to be implemented via cyclin D1, whereas activated ER induces cyclin D1 expression by an as yet unknown mechanism; it seems clear that ER-mediated proliferation is implemented via induction of cyclin D1. The activation of ER by cyclin D1 and its consequences were discussed in detail. In conclusion, activation of ER lead to induction of cyclin D1 expression, and excess of cyclin D1 activates and stimulates ER-dependent effects; overactivation of cyclin D1 in transgenic mice leads to mammary gland hyperplasia and neoplasia, the latter in cooperation with other oncogenic changes. The interactions between cyclin D1 and ER may provide exploitable sites for novel therapeutic intervention.

As reported by Ferrari, a member of the myb proto-oncogene family, B-Myb, may play an important role in late G1 and early S phase of the cell cycle. B-Myb undergoes cyclin A/Cdk2 complex-mediated phosphorylation at the onset of S phase, and this triggers its transactivation potential; this activation is in contrast with the reported inhibition of E2F-DP after phosphorylation by that complex. This was the first reported evidence for a positive role of cyclin A/Cdk2 and a possible function of B-Myb in early S-phase.

John Reed reviewed the molecular mechanisms involved in the function of Bcl-2 and the dysregulation of PCD in cancer. To date, 15 cellular homologs of Bcl-2 have been found, some of them, like Bcl-XL, with antiapoptotic activity, some, like BAX, with proapoptotic activity; 5 homologs have been found in viruses. Both antiapoptotic and proapoptotic proteins can homodimerize or heterodimerize with members with opposite function. Other apoptotic-promoting proteins, like Bad, act by sequestering antiapoptotic proteins like Bcl-2 or Bcl-XL; Bid is a proapoptotic protein that can dimerize with both pro- and antiapoptotic proteins but not with itself, thus exhibiting unique dimerization properties. It seems that at least some Bcl-2 proteins exist in two conformations, one creating a receptor-like pocket and the other exposing the hydrophobic surface of the α-helix, which would link in the receptor-like pocket of the dimerization partner; four Bcl-2 homology domains are important for function, as demonstrated for Bcl-2 homology domain 3. Despite the many advances made, the functional significance of several of these protein-protein interactions is still controversial. For example, the role of Bax homodimerization in apoptosis induction is still unclear. In some types of solid tumors, like prostate cancer, the expression of the Bcl-2 gene becomes dysregulated during progression. Relatively less is known about the expression of other genes of the Bcl-2 family in human tumors; however, Bcl-XL has been found in many colorectal cancers, and its expression tends to be reciprocal regulated with that of Bcl-2. The regulation of Bax and Bak in human cancer was also discussed. The initiation, commitment, and execution phases of the PCD pathway were outlined; the Bcl-2 family proteins appear to control the commitment phase of the process and to determine whether certain caspases will be activated. However, pathways to bypass Bcl-2 exist as those activated by certain members of the tumor necrosis factor family receptors, e.g., Fas/APO-1 (CD95); these pathways can also be blocked by inhibitors like FAP-1 or FLIP. The molecular events representing the decision of a cell to commit to PCD or to survive despite exposure to apoptotic signals are still obscure; the possible role of mitochondrial permeability transition in this decision was discussed. Mitochondrial permeability transition occurs early during apoptosis; Ca\(^{2+}\) release into the cytosol may induce Ca\(^{2+}\)-dependent protease activation, and release of apoprtotic proteins like cytochrome C and apoptosis-inducing factor can lead to caspase activation. The possibility that Bcl-2, which is located at least in part in the outer mitochondrial membrane, regulates mitochondrial megapore is supported by several findings. The Bcl-2 family proteins also have a function as adapter/docking proteins, but in most cases the functional significance of these interactions is still obscure. Bcl-2 overexpression can prevent p53 translation from cytosol to nucleus; the ability of Bcl-2 to bind calcineurin, sequestering it at membranes, may also be instrumental in the antiapoptotic effect of Bcl-2. Post-translational modifications of Bcl-2 family proteins through phosphorylation have been described. The potential of Bcl-2 to modulate cell proliferation and the possible role of interactions with transcription factors such as nuclear factor-xB and nuclear factor-AT have been studied. Finding strategies to overcome the cytoprotective effects of Bcl-2 represent a valid approach to therapeutic intervention. Indeed, the genetic instability occurring in many tumors favors apoptosis, if it were not for the antiapoptotic action of Bcl-2 family proteins.

**Tumor Antigens and Immune Responses**

As discussed by Coulie, based on their patterns of expression, tumor antigens can be placed into four groups: group 1, antigens encoded by genes expressed in tumor cells but not in normal cells, except male germinal cells; group 2, differentiation antigens expressed in melanoma and normal melanocytes; group 3, antigens unique to individual tumors consequent to tumor-specific mutations; and group 4, antigen encoded by genes overexpressed in tumor cells.
Using a genetic approach, a number of genes for melanoma antigens were cloned; these antigens consist of a peptide derived from an intracellular protein and presented by an HLA class I molecule. In malignant melanomas, examples of antigens of the first group are MAGE, BAGE, or GAGE. The fact that they are shared by different tumors makes them potentially useful for immunotherapeutic intervention. The MAGE gene family was discussed in detail; MAGE-I is present in 48% of metastatic melanoma as well as in a number of breast tumors, non-small cell lung carcinoma, sarcomas, bladder carcinomas, and head and neck carcinomas. In studies of antigenic diversity, it was found that different peptides from the MAGE-I protein can bind to different HLA class I molecules within the same cells and thus form two antigens recognized by different CTLs. MAGE-3 is more frequently expressed in melanoma than MAGE-1 and thus lends itself better to immunotherapy utilization. Using similar approaches, a new antigen coded by a gene named RAGE was identified on several renal cell carcinoma lines; this gene is silent in normal tissues except the retina. Because the retina does not express MHC class I molecules, this antigen seems to be tumor specific; it is also expressed in a small proportion of sarcomas, bladder tumors, and melanomas. Antigen NA17-A is coded by a gene expressed ubiquitously, that of N-acetyl-glucosaminyltransferase V, which has, however, a promoter activated only in melanoma cells. Among the different antigens found on melanoma cells, notable are tyrosinase, Melan-A/MART-1, gp100, and gp75; most of them are presented by HLA-A2. The presence of these antigens in many patients makes them also possible candidates for immunotherapy exploitation. Unique antigens derived from point mutations of a variety of genes have been identified; these may range from cdk4 to -catenin to caspases, to HLA-A2, and others. Antigen uniquely expressed would be specific for a given individual, and this makes it difficult to use them for immunotherapy. An antigen resulting from overexpression of genes in a melanoma clonal cell line was coded by a gene named PRAME; this gene was found to be expressed in a large proportion of different types of tumors including acute leukemia. Characterization of the antigen CTL17 indicated that CTL 17 may represent a new type of antitumor lymphocytes, showing specificity for tumor cells that have lost expression of some HLA class I molecules. In initial clinical studies, 5 of 17 patients with advanced melanoma showed tumor regression after several s.c. injections of a peptide encoded by MAGE-3 and presented by HLA-A1.

Michael Pfreundschuh discussed the identification of human tumor antigens using the B-cell repertoire. Indeed, several molecules in tumor cells have been shown to elicit humoral responses in cancer patients, for instance Her2/neu, p53, ras, E6 and E7, c-myc, c-myb, and MUCI. A new technology named SEREX, namely serological analysis of tumor antigens by recombinant cDNA expression cloning, was developed applying molecular cloning methodology to the autologous typing approach. The SEREX methodology uses fresh tumor specimens and thus avoids artifacts related to in vitro culturing. Moreover, the spectrum of expression of an antigen can be identified by Northern blots and reverse transcription-PCR. At least four different antigens have been found in each tumor; more than 100 different human tumor antigens have been identified, among which were also known CTL-recognized melanoma antigens. A second group of antigens consists of transcripts of known genes that had not been known to elicit immune responses in humans, for example, restin. A third group is represented by antigens expressed by previously unknown genes. Characterization studies revealed different types of antigen specificity. A new class of cancer/testis antigens was defined. The expression of these antigens is favored by the genome hypomethylation characteristic of cancer cells and tests. The clinical significance of antitumor antibodies is unknown in most cases.

The tolerance of T cells toward tumor or in autoimmunity was reviewed by P. Ohashi. Thymic tolerance can occur by clonal deletion in the presence of certain superantigens or conventional or peptide antigens. Because peptide vaccines are used in immunotherapy studies, it is important that they are not derived from a protein abundant in thymus. It has been suggested that tolerant unresponsive thymic cells may be responsible for the lack of response. Peripheral tolerance has been proposed to be related to clonal deletion of tumor-specific T cells as the tumor mass grows and the associated antigens become more abundant. A state of unresponsiveness can develop in the presence of antigen after an initial period of responsiveness. Another mechanism is related to down-regulation of TCR or decrease of density of CD8. In the absence of CD 28 and B7-1 costimulatory signals, T cells do not respond, but in contrast to previous suggestions, they do not become permanently anergic in the presence of normal TCR functions. In another model, self-reactive T cells exist but remain naive and may be induced to acquire full effector function. The mechanisms of unresponsiveness alluded to above should be viewed in the light of evidence that in vivo T cells are not tolerant to many tumor-associated antigens. In transgenic RIP-Tag2 mice expressing SV40 large T antigen in the pancreatic β islets, islet tumor develops at 8–16 weeks of age; using this model, evidence was obtained that T-cell tolerance did not develop but that multiple stimulation may be required to retain immunosurveillance in vivo and to develop memory cells.

Jim Allison discussed possibilities of modifying T-cell activation in antitumor immune responses, also within the context of developing effective cancer immunotherapy. Activation of T cells depends on two signals through different receptors, TCRs engaged by antigenic peptides bound to MHC molecules and CD28 on T cells binding to B7 on antigen presenting cells (APCs). This activation includes cytokine production, clonal expansion, and functional differentiation of antigen-specific T cells. Only professional APCs like dendritic cells, B cells, and macrophages express both antigenic peptide-MHC complexes and costimulatory B7 molecules. CTLA-4 is also induced after complete activation; it binds avidly to B7, and it down-regulates T-cell responses. As a consequence, blockade of CTLA-4 would enhance these responses. Tumors transduced to express B7 appeared to act as APCs to prime CD8+ T cells and were in some cases rejected. Poorly immunogenic tumors, transduced with B7, could not be rejected unless also transduced with IFN-γ; similar results were obtained by cotransduction of B7 and IL-12 but not of B7 and GM-CSF. Thus, synergy is best when similar pathways of T-cell activation are augmented. In a different approach, antibody blocking the CTLA-4/B7 interaction led to rejection of murine colorectal tumor 51Blim10 transfected with B7. Verification using several tumor models indicated that this approach is effective in some, but not all, tumor systems; in more immunogenic tumor models, anti-CTLA-4 treatment was effective even when delayed until a substantial tumor burden was measured. Although the mechanisms of the anti-CTLA-4-dependent tumor rejection is not yet clear, it is certain that the effect is dependent on host-derived APCs. Synergy was noted when some tumors were transduced to secrete GM-CSF and were treated with anti-CTLA-4 antibody, supporting the hypothesis that APCs were recruited that could prime both CD4+ and CD8+ T cells. Anti-CTLA-4 antibody was also effective in combination with whole tumor cell vaccines in studies with poorly immunogenic B16 melanoma. The approaches to enhance activation of T cells by blockade of CTLA-4 alluded to above provide a good basis for the development of immunotherapies of cancer.

G. Forni discussed the potential therapeutic exploitation of defined tumor vaccines designed taking advantage of the increased knowledge of antigen presentation and T-cell activation processes. In contrast
with classical preventive vaccination, “therapeutic vaccination” is applied when the tumor is already present and growing. Recent progress in genetics and tumor predisposition may open the way toward implementing preventive vaccination also in the case of tumors. The capacity of tumors to evade immune responses is often related to an insufficient processing and presentation of TAA, the absence of adhesion molecules or costimulatory signals, the hindered recruitment of APCs, the tumor-induced Fas-related apoptosis of activated lymphocytes, and/or the release of an array of immunoderegulating cytokines. Consequently, any antigenic signal from the tumor is ignored or insufficient. Vaccines designed to enhance TAA immunogenicity can exploit rational genetic engineering approaches aimed at inserting new genes into the tumor genome. Tumor cells can be engineered to express costimulatory molecules, typically B7, such that direct presentation of TAA to specific lymphocytes becomes efficient. This approach is possible with immunogenic tumors or tumors rendered immunogenic by suitable transfection. Because the immunogenicity of tumors also depends on adequate adhesion of tumor cells to lymphocytes, transduction of both B7 and ICAM-1 leads to improved T-cell recognition and establishment of memory. Additional factors involved in the tumor rejection seen under these circumstances are related to the inflammatory responses. Tumor cells can also be transduced with MHC class I genes, thus obviating the MHC deficiencies found in some cases. A mirror image approach is to genetically engineer dendritic cells to express TAA or related peptides constitutively; an analogous approach is to fuse dendritic cells with tumor cells, thus encompassing a large number of antigens. Vaccination by inoculating DNA, including gene sequences encoding for TAA, is also being pursued. Tumor cells engineered to secrete relevant cytokines in the tumor microenvironment led to augmented local antitumor reactions. The specificity of the effector cells was determined by the cytokine released. Tumor-specific, T-cell-dependent mouse memory is induced through mechanisms including loading the system with sufficient tumor antigen, intervention of appropriate APC, and presence of the cytokine; this memory is consequent to tumor regression, no matter how it is brought about.

C. J. M. Melief reviewed present knowledge of the antitumor effects of NK cells and CTLs in tumor immunity. NK cells recognize target cells by their receptors and kill them unless inhibited by messages from killing inhibitory receptors that recognize MHC class I domains or more broadly HLA.s. Through the actin of these inhibitory receptors, normal untreated cells, most of which express MHC class I molecules, are protected from NK cell killing. Tumor cells tend to down-regulate MHC class I molecules and become more sensitive to NK cells. Each CTL recognizes a specific peptide-MHC complex through the combination of the α and β chains of the TCR; CTLs are positively selected in the thymus, and more than one-half of them are clonally deleted. Unstimulated CTLs that leave the thymus are activated upon stimulation, mainly by dendritic cells and monocytes presenting antigen. The expression of B7.1 (CD80) and B7.2 (CD86) is essential inasmuch in their absence TCR engagement can result in anergy or apoptosis. Upon activation and recognition of a peptide-MHC complex, target cell lysis occurs. Some CTLs also express killing inhibitory receptors. Both NK cells and CTLs can be activated with cytokines such as IL-2. Because of the specificity of CTL activation by peptide-MHC complexes, peptides have been used for vaccination. Large proteins can be screened for peptides that fit the binding motif of certain MHC molecules. Because naked peptides can sometimes induce tolerance, alternative vaccination strategies are attempted such as those using DNA encoding specific antigenic moieties or engineered viral vectors; cytokines such as IL-12 and GM-CSF can amplify the effects obtained. The process of tumor antigen presentation was outlined. Tumor-specific antigen derived from mutations of normal proteins or oncogenes have limitations for vaccine preparation based on their restriction to individuals and the laborious isolation and purification involved. Tissue-associated antigens such as gp100 or MART-1 are not unique to tumors but, based on differential expression and/or recognition, can be used for antitumor vaccine preparation. Normal antigens overexpressed in tumors, like p53, HER-2/neu, mdm2, or CEA, have also provided a basis for vaccine preparation. Tumors can escape immune responses by secreting inhibitory cytokines, like transforming growth factor β, or down-regulating their MHC class I molecules, or by losing antigen expression.

S. Evans pointed out the importance of immune cell migration into tumors for an effective antitumor immune response. Effector cells extravasate across postcapillary endothelial venules (HEVs); L-selectin mediates the initial attachment and slow rolling of lymphocytes along HEVs under hemodynamic shear conditions; indeed, monoclonal antibody blockade of this molecule eliminates antitumor immune responses in animal models. Firm adhesion of lymphocytes to HEVs and transendothelial migration depends on the interaction of leukocyte function associated antigen-1 with ICAMs (ICAM-1 and ICAM-2). The αβ1 and αβ2 integrins are also involved through binding to their cognate receptors on endothelium, including vascular cell adhesion molecule-1 and mucosal addressin cell adhesion molecule-1. Blockage of these leukocyte-endothelial cell adhesive interactions compromises antitumor immunity as naive, and memory CD4 and CD8 T cells, monocytes, neutrophils, and NK cell subsets cannot reach the target area. Tumors interfere with the mechanisms of effector cells extravasation, and tumor-derived basic fibroblast growth factor and transforming growth factor β suppress adhesion molecules. Antitumor immunity may be enhanced through the regulation of adhesion molecules. It was found that IFN-α regulates L-selectin gene expression and cell surface levels in human lymphocytes: increased transcriptions, mRNA levels, and surface expression were noted. Transcription of L-selectin was activated by IFN-α in IFN-sensitive but not in resistant cells through a signaling pathway close to the JAK/STAT pathway. A region of the L-selectin gene upstream of the transcript initiation site has been identified that confers IFN-α responsiveness onto a heterologous reporter gene. The hypothesis was discussed that L-selectin gene in hematopoietic cells is transcriptionally activated through interactions of IFN-α-inducible STAT proteins and non-STAT transcription factors with cis-acting elements in the L-selectin promoter. Lymphocyte activation is accompanied by loss of L-selectin via shedding; in vitro certain cytokines, like IL-12 and IFN, increase L-selectin on NK cell subsets and can also maintain L-selectin expression during lymphocyte activation. Shedding of L-selectin was found to be controlled by the M16,000 IFN-inducible Leu 13 protein. This protein is constitutively expressed at high levels on B and T lymphocytes. Antibodies binding to Leu 13 inhibit growth factor-driven proliferation of normal and malignant lymphocytes. Through Leu-13 cloning, it was found that it is encoded by the IFN-α-responsive 9-27 gene and that the transcription factor ISGF3 is involved in its transcription. Signaling via Leu 13 leads to down-regulation of L-selectin on B and T cells through increased shedding. It was found that Leu 13 initiates a novel tyrosine kinase-dependent/protein kinase C-independent signal transduction pathway regulating this shedding. It appears that Leu 13, like PMA, initiates L-selectin shedding through a zinc-dependent metalloproteinase. More recently, it was found that hyperthermia and proinflammatory cytokines increase L-selectin-mediated leukocyte-endothelial cells adhesion. Hyperthermia increased the avidity of preexisting L-selectin for physiological ligands, in parallel with a marked increased in L-selectin associated with cytoskeleton matrix; these effects are mediated by soluble factors secreted by the heated cells, which are being identified. Additionally,
it was found that hyperthermia also acts on the endothelium and increases L-selectin-mediated lymphocyte-endothelial cell adhesion through increases of L-selectin avidity for ligands. It is therefore possible that hyperthermia results in increased recruitment of immune effector cells in vascularized tumor sites and regional lymph node through the regulation of L-selectin-dependent adhesion.

Investigations of the migratory behavior of murine dendritic cells, human melanoma cells (MV3), and human T lymphocytes in a three-dimensional collagen matrix were outlined by Entschladen. T cells were fastest, dendritic cells were slower, and MV3 cells were the slowest. Using anti-β1 integrin monoclonal antibodies, it was demonstrated that T cell and dendritic cell migration is independent of β1 integrin mechanisms. In contrast, MV3 cells release β3 integrins from the trailing edge, supporting a prior cell adhesion process. Also, based on the distribution of focal adhesion kinase and protein kinase C, it was concluded that migration of T lymphocytes does not involve local adhesion processes.

Angiogenesis

P. Carmeliet outlined the basic mechanisms of blood vessel formation, mainly in embryogenesis, with emphasis on gene targeting as an approach useful for the identification of the factors involved. Initially, the blood vessels are formed as endothelium-lined channels consequent to in situ differentiation of endothelial cells; subsequently, they remodel into an organized and interconnected network; furthermore, primitive vascular smooth muscle cells surround the channels. The VEGF is produced in close vicinity of endothelial cells, it interacts with endothelial tyrosine kinase receptors Flk-1, Flt-1, and Flt-4, its expression is regulated by hypoxia, and it has potent activity on cell growth. Several isoforms of VEGF have been identified. Another tyrosine kinase receptor family is TIE; angiopoietin-1 has been identified as the ligand of TIE-2/TEK, a receptor affecting periendothelial cells function. Receptor TIE-1 does not seem to be involved in early endothelial cell differentiation but seems to be required for the structural integrity of the microvascular endothelial cells. After the endothelial cell channels are formed, they become surrounded by smooth muscle/pericytes. Evidence has been obtained that certain factors of the coagulation system may be involved in other functions beyond hemostasis, including cellular migration and proliferation, and appear to be related to the development and/or maturation of mesenchymal cells and, consequently, the formation of vascular pericytes. The possible mechanisms involved in these functions were discussed in detail. Angiopoietin-1 has been identified as the ligand of TIE-2/TEK and to induce phosphorylation of this receptor. Targeting the angiopoietin-1 gene led to abnormal cardiovascular development and death around day 12; the evidence available suggests that remodeling of the initially homogeneous capillary network into large and small vessels was defective and possibly associated with a failure in recruitment of periendothelial mesenchymal cells. Blood vessel formation can also be affected by transforming growth factor β, which seems essential for vascular integrity and maturation of the vascular bed. The platelet-derived growth factor PDGF-BB is angiogenic in vivo but is not clear whether this effect is mediated through recruitment of inflammatory/mesenchymal cells or direct action on endothelial cells. Taken together, it appears that PDGF-BB plays a central role in providing the structural integrity of the maturing vasculature.

The role of VEGF in tumor angiogenesis was discussed by G. Breier. Tumor growth beyond the stage of small avascular solid tumors requires neovascularization, which would be able to meet the growing tumor demands for oxygen and nutrients. The switch from prevascular-phase to vascular-phase tumors seems to result from a balance between endogenous stimulators and inhibitors of angiogenesis. A number of angiogenic growth factors have been identified, including fibroblast growth factors and VEGF. VEGF has multiple functions in normal angiogenesis that have been verified in tumors; the specific signaling pathways used by the VEGF-receptor systems are incompletely characterized. In tumors, VEGF has a major angiogenic role in vivo. For example, in gliomas VEGF seems to function as an angiogenesis factor. Angiogenesis accompanies tumor progression from low to high grade. VEGF mRNA is up-regulated in certain tumor areas, and VEGF receptors are induced in the tumor vasculature, suggesting a paracrine function of VEGF. The VEGF/VEGF receptor systems are up-regulated in a variety of human tumors, including breast carcinoma, where a correlation between blood vessel density and VEGF levels have been demonstrated. Hypoxia and hypoglycemia up-regulate VEGF expression in vitro through both transcriptional activation and increased mRNA stability; the former proceeds through binding of hypoxia-inducible factor-1 to regulatory cis-acting sequences in the 5’ flanking region of the VEGF gene, and the latter is mediated by 3’ untranslated sequences. It was demonstrated that these mechanisms also occur in vivo. Further analysis indicated that oncogenic transformation and the microenvironment cooperate in stimulating VEGF expression and tumor angiogenesis. In mouse mammary carcinoma, it was found that V-Ha-Ras transformed cells from rapidly s.c. growing tumors in nude mice. Angiogenesis was accompanied by VEGF and VEGF receptor expression in the tumor vasculature, and VEGF expression was synergistically up-regulated by Ras and hypoxia. TGF-β up-regulated VEGF both in nontumorigenic and Ras-transformed tumorigenic epithelial cells in vitro, in cooperation with hypoxia, and it was found that high levels of VEGF expression per se are not sufficient to induce tumor growth, indicating that VEGF does not act as an oncogene. The VHL tumor suppressor gene acts as a negative regulator of VEGF expression; in hemangioendolastoma, mechanisms other than hypoxia lead to VEGF up-regulation. In additional studies, it was found that Flk-1 is up-regulated by VEGF in brain slices in vitro and that this effect could be inhibited by the addition of neutralizing anti-VEGF antibodies. In conclusion, it would seem that VEGF signal transduction may represent a useful target for antiangiogenesis therapy.

R. K. Jain discussed the phenomenon of conditioning the transfer of molecules, particles, or cells from blood to tumor cells, i.e., distribution through the vascular compartment, transport across the microvascular wall, and transport through the interstitial compartment. For a given molecule, these processes may involve diffusion and convection. In addition, a molecule may bind nonspecifically or specifically or be metabolized. Cells are capable of deformation but are still restricted in their motion by the same barriers. The discussion concerned: how angiogenesis takes place, what determines blood flow heterogeneity in tumors, how blood flow influences the metabolic microenvironment of tumors and how this microenvironment affects the biological properties of tumors, how materials move across the microvascular wall, and how it moves through the intestinal compartment and the lymphatics. The role of cell deformation and adhesion in delivery of cells was also considered. Five approaches were pursued to study the pathophysiology of solid tumors: (a) a tumor that is connected to the host circulation by a single artery and a single vein; (b) rabbit ear, mouse dorsal chambers, and a cranial window in mice and rats; (c) in vitro measurements of deformability adhesion and permeability of normal and neoplastic cells; (d) routine molecular biology techniques; and (e) mathematical models to integrate the results obtained. Although the tumor vasculature originates from host vasculature through similar basic mechanisms, its organization may be different, depending on tumor type, and governs the movement of molecules. For example, in tumors, unlike in normal...
tissues, red blood cells (RBC) velocity is not dependent on vessel diameter and the temporal and spatial heterogeneity in tumor blood flow may be affected by elevated geometric resistance in tumor vessels, coupled with vascular permeability and elevated interstitial fluid pressure as well as vascular remodeling. In a tumor four regions can be identified, namely an avascular and necrotic region, a seminecrotic region, a microcirculation region and the advancing front. By using fluorescence ratio-imaging microscopy and phosphorescence quenching microscopy, $pO_2$ and $pH$ were measured in different areas of tumor. The transport of a blood-born molecule across the microvascular wall is affected by physiochemical parameters that were discussed in the light of the fact that vascular permeability and hydraulic conductivity of tumors in general is higher than that of several normal tissues. The paradoxical aspects of poor extravasation of molecules through leaky tumor vessels may be related to the high interstitial pressure in tumor and the balance between intravascular and extravascular pressures. After extravasation, the movement of a molecule through the interstitial fluid pressure in different areas of the tumor and radial outward velocity affect the capacity of a macromolecule at the periphery of the tumors to diffuse into it. Because of lymphatics within the tumor, materials oozing from the tumor surface must be picked up by peritumor host lymphatics. The transport of cells into tumors is affected by both local hydrodynamic and adhesive forces, the former determined by vessel diameter and fluid velocity, and the latter by adhesion molecules and contact surface areas. Remodelability of cells affect both sets of factors. Certain cytokines, for example, IL-2, impart rigidity to A-NK cells, which favor their entrapping in lung. In contrast to VEGF, bFGF has no effect per se but eliminates the up-regulation induced by VEGF on TNF. The various steps in the delivery of molecules and cells to and within tumors have been interpreted using pharmacokinetic modeling.

In conclusion, this Symposium was focused on the molecular and cellular phenomena underlying specific aspects of the biology of tumors, *i.e.*, genetic factors determining the pathogenesis and progression of tumors, the genetic control of the cell cycle and of apoptosis in programmed cell death, the role of tumor immunity in tumor pathogenesis and the opportunities for intervention, the mechanism of angiogenesis in normal and tumor tissues, and the possibility of developing specific antiangiogenesis-based tumor therapeutics. Although much needs to be done in each of the above areas of research, remarkable progress has been achieved, which was the basis for exciting and stimulating discussions.
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