

Meeting report

Revealing the intricacies of cancer

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A report on the 14th Lorne Cancer Conference, Lorne, Victoria, Australia, 14-17 February 2002.

The 'guardian of the genome', the p53 protein, a key regulator of cell cycle arrest and apoptosis, featured prominently at this year's Lorne Cancer Conference. The plenary seminar was presented by David Lane (University of Dundee, UK), whose research has been central to the transformation of p53 from being considered a weak oncogene to its now clearly demonstrated role as a powerful tumor suppressor. There were also several reports on pathways mediated by another tumor suppressor, the retinoblastoma protein (pRb) as well as updates on an ever-increasing array of proteins and signaling pathways implicated in cancer. We learned of several animal models for cancer and how they may be used to screen for as-yet unidentified oncogenes or tumor suppressors; and we heard about attempts to develop cancer vaccines, p53-targeted tumor therapy, and the use of metallo-proteinase inhibitors in cancer treatment. The following are some of the highlights.

Upstream and downstream of p53 and pRb

The activation of p53 in response to DNA-damaging agents and other forms of stress is part of a protective mechanism that ensures cell-cycle arrest and DNA repair or, alternatively, apoptosis. Many tumor cells lack p53 function, and it seems there is enormous potential for therapy based on restoring p53 function, either directly or by inhibiting the E3 ubiquitin ligase MDM2 that regulates p53 degradation. By switching on p53, therapies would selectively assist tumor cells - but not healthy cells - to undergo apoptosis, because the tumor cells are less responsive to normal environmental survival signals and are generally exposed to more apoptotic stress (for example, hypoxia and oncogenic activation). The

importance of establishing the exact p53 status of the tumor before considering p53-based tumor therapies was emphasized several times at the conference, however. For example, high levels of mutant p53 in cancerous cells could impede the function of introduced wild-type p53 protein, if the mutant protein proves able to act in a dominant-negative fashion.

The plenary talk by Lane focused on how p53 function might be restored to tumor cells, emphasizing that in many cases the p53 gene is not mutated but there is a block in a pathway(s) affecting p53. The MDM2 protein featured as a key target for therapy; in this instance, small peptides that block the p53-MDM2 interaction or that block MDM2 activity could serve to activate wild-type p53. Indeed, only the first 16 amino acids of a natural inhibitor of MDM2, the p14^{ARF} protein, are necessary for p14^{ARF} to interact with MDM2. In cervical cancer, p53 is targeted for degradation by the E3 ligase activity of the human papillomavirus (HPV) 16/18 E6 protein. The drug leptomycin B protects p53 from degradation by E6 or MDM2, probably by blocking nuclear export of p53 and, when used in combination with a second drug, actinomycin D (to inhibit E6 mRNA expression), has the potential to increase p53 levels further. Although leptomycin B has been found to be toxic in clinical trials, lower doses could still prove to be effective in the treatment of tumors that have wild-type p53, in particular in HPV-associated cervical cancer, where topical application could be used. For treatment of tumor cells lacking wild-type p53, Lane proposed mimicking the activity of p53 target genes (for example, p21) as a more appropriate approach.

The p53 protein acts as a transcription factor - but is that all it does? Karen Vousden (National Cancer Institute, Fredrick, USA) revealed what she thinks are the capabilities of p53. Although everyone agrees that p53 can induce the transcription of proteins involved in DNA repair (for example, p53R2 and Gadd 45), in cell-cycle arrest (for example, p21^{CIP} and

14-3-3) and in apoptosis (for example, PUMA, Noxa and p53AIP1), its apparent ability to induce apoptosis independent of transcription was not expected. The evidence for this independence comes from experiments using a truncation mutant of p53 (Tr210) that is missing the carboxy-terminal oligomerization domain and much of the DNA-binding domain. Tr210 is unable to induce the transcription of several known p53-inducible genes but can, nevertheless, induce apoptosis. Because Tr210 lacks the oligomerization domain, it cannot be overcome by the dominant-negative effect of p53 mutant proteins, that are often highly expressed in cancer cells, and it is also not targeted for degradation by either of the E3 ubiquitin ligases, MDM2 or the viral protein E6. The pro-apoptotic function of Tr210 was mapped to a 37 amino-acid region, raising the possibility of peptide-based tumor therapy. Given that many tumors have lost their ability to stabilize p53, Vousden has begun screening for specific inhibitors that would target the E3 ubiquitin-ligase activity of MDM2 but not that of other E3 ligases.

As it enhances p53 stability, p14^{ARF} is receiving much attention. Intriguingly, p14^{ARF} is encoded by a locus that also specifies p16^{INK4a}, a distinct polypeptide that has attracted equal attention, but this time by virtue of its ability to promote pRb function. Whereas p14^{ARF} acts by inhibiting MDM2-mediated ubiquitination and degradation of p53, p16^{INK4a} inhibits phosphorylation mediated by cyclin-dependent kinase and inactivation of pRb. Gordon Peters (Cancer Research UK, London, UK) has found that dermal fibroblasts from certain melanoma-prone individuals have inherited mutations in both alleles of the *INK4a/ARF* locus that result in loss of expression of p16^{INK4a} but no effect on p14^{ARF} function. The cells are insensitive to growth arrest mediated by the Ras GTPase (a proto-oncogene), and Ras expression allows cells immortalized by over-expression of telomerase to form anchorage-independent colonies in soft agar, although it does not facilitate the growth of these cells as tumors in nude mice, suggesting that additional genetic modifications are required. David Thomas (University of Melbourne, Australia) has found a role for pRb in osteoblast differentiation and cell-cycle exit that relies on the ability of pRb to interact with the osteoblast transcription factor CBFA1, shedding light on the observation that mutation in the *Rb* gene commonly leads to osteosarcoma formation.

Two melanoma-associated mutations of the *INK4a/ARF* locus, described by Helen Rizos (University of Sydney, Australia), specifically affected the sequence of p14^{ARF} but not of p16^{INK4a}, resulting in cytoplasmic and nucleoplasmic expression of p14^{ARF} rather than nucleolar expression, and a reduction in the capacity of p14^{ARF} to activate the p53 pathway. Searching for proteins that interact with the ARF protein, Renu Wadhwa (Chugai Research Institute for Medical Sciences, Ibaraki, Japan) has identified a protein, Pex19p, that interacts with the carboxyl terminus of p19^{ARF} and appears to relocate p19^{ARF} from the nucleus into the cytoplasm,

negatively regulating p19^{ARF} function. One caveat, however, is that Pex19p does not interact with human ARF (p14^{ARF}). Moshe Oren (The Weizmann Institute, Rehovot, Israel) presented studies on β -catenin, a transcription factor and structural component of adherens junctions that becomes activated in colorectal cancer. He showed that β -catenin induced the expression of p14^{ARF} (human) and p19^{ARF} (mouse), thereby activating p53, whereas in cells lacking p53 the potential of β -catenin as an oncogene was realized.

There has been much hype about the potential use of adenoviruses in cancer therapy. One such virus, ONYX-015 is now in phase III clinical trials for the treatment of head and neck cancers. In a talk by Antony Braithwaite (University of Otago, Dunedin, New Zealand), however, we were forced to reconsider the usefulness of ONYX-015 as a cancer treatment. In theory, ONYX-015, which lacks the *E1b55k* gene (the product of which binds and inactivates p53), should only replicate in (and kill) cells that do not have functional p53 protein (that is, tumor cells). In Braithwaite's studies, however, ONYX-015 showed no selectivity for p53-defective cells, nor indeed for most tumor cells, and it was in fact highly attenuated in its capacity to infect and replicate in any cell type.

Genetic susceptibility to cancer

The RecQ helicase family plays important roles in DNA repair. Mutations in genes of this family result in genomic instability and increased susceptibility to cancer. People with Bloom's syndrome carry mutations in the *BLM* gene, and fibroblasts derived from these individuals are resistant to p53-mediated apoptosis. Curtis Harris (National Institutes of Health, Bethesda, USA) showed that, in response to ionizing radiation, up to 90% of BLM becomes associated with p53, and that p53 is able to modulate the co-localization of BLM with promyelocytic leukemia (PML) bodies in the nucleus and to disrupt BLM-mediated disruption of Holliday junctions - the branch point of crossed DNA strands undergoing repair or recombination. The carboxyl terminus of p53 mediates the interaction, and carboxy-terminal peptides of p53 are able to inhibit both BLM and another RecQ helicase, WRN, and to induce apoptosis. Phosphorylation of p53 by protein kinase C prevents the interaction of p53 with BLM and WRN.

The disease ataxia telangiectasia (A-T) is characterized by increased radiation sensitivity and a predisposition to leukemia and lymphoma. A-T occurs in people lacking ATM, a protein kinase that is activated by ionizing radiation and that protects cells from chromosomal damage. Individuals with a single mutant *ATM* allele are also susceptible to cancers (for example, breast cancer). Martin Lavin (Queensland Institute of Medical Research, Brisbane, Australia) reported that the most common *ATM* mutation in humans is a deletion resulting in the loss of three amino

acids, Ser-Arg-Ile, from the ATM protein. To explore the significance of this deletion, a 'knock-in' mutation was generated in the mouse. Animals heterozygous for this mutation developed tumors, whereas heterozygotes with one normal *ATM* allele and an *ATM*-null allele did not. This suggests that the product of the mutant allele may interfere with the function of the wild-type ATM protein and, indeed, when introduced into cells, the mutant ATM protein abolished radiation-induced activation of the ATM kinase, inhibited cell survival and increased the occurrence of chromosomal aberrations. Although the precise mechanism remains to be elucidated, these studies indicate the potential for mutant forms of ATM to act in a dominant negative manner in mice and humans.

Von Hippel-Lindau (VHL) syndrome is a rare inherited disease in which individuals with defects in the *VHL* gene develop blood vessel tumors in the retina and central nervous system, renal carcinomas and pheochromocytomas (adrenalmedullary tumors). A major function of the *VHL* gene product, the pVHL protein, is to promote the ubiquitin-dependent degradation of a hypoxia-inducible transcription factor, HIF. In VHL syndrome, the absence of pVHL leads to increased stability of HIF and enhanced expression of its target genes - including those for the angiogenic factor, VEGF (vascular endothelial growth factor). William Kaelin (Harvard Medical School, Boston, USA) showed that pVHL and HIF only interact after an intriguing post-translational modification in which a key proline residue within HIF is hydroxylated in an iron- and oxygen-dependent manner. HIF mutants lacking this proline can escape pVHL-dependent degradation and promote tumor formation even in the presence of pVHL. Thus, pVHL appears to function as an O₂ sensor in normal cells; it prevents HIF overexpression and subsequent downstream events such as angiogenesis.

Cancer models

Our understanding of cancer has greatly benefited from the generation of mouse models, although they have their limitations. Tyler Jacks (Massachusetts Institute of Technology, Cambridge, USA) reported on a mouse conditional lung carcinoma model, in which adenoviral delivery of the Cre recombinase enzyme to the lungs of genetically targeted animals carrying *loxP* insertion sites (recognized by Cre) results in the activation of an oncogenic *K-Ras* allele. Tumors develop four weeks after infection with adenoviral vector, even at the lowest doses of virus tested, and result in multiple tumors per mouse. Simultaneous K-Ras activation and expression of a dominant-negative mutant p53 resulted in metastatic tumors similar to those seen in man.

The adenoviral Cre-recombinase model has allowed study of different stages of lung cancer. In addition to papillary adenoma (positive using antibody to human surfactant protein C) and bronchiolar adenoma (positive using antibody

to Clara cell antigen), a third type of tumor, (positive for both these markers) was detected, suggesting either trans-differentiation of tumor cells or expansion of a rare stem cell. Another focus of Jacks' talk was pRb and its role in tumorigenesis. Examining *Rb*^{+/-} mice it was found that 100% get pituitary tumors and 50% get thyroid tumors but, unlike humans of this genotype, none develops retinoblastoma. This highlights a limitation of this mouse model for human cancer. It is possible that in the mouse model there is functional compensation by pRb family members (for example, p107 and p130). Indeed, elimination of both pRb and p107 was required for retinoblastoma to develop. Cultures of *Rb*^{-/-} mouse embryonic fibroblasts (MEFs) were found to undergo senescence in an *in vitro* growth assay whereas Triple Knockout MEFs (*Rb*^{-/-}, *p107*^{-/-}, *p130*^{-/-}) were immortal. Intriguingly, deletion of *Rb* alone from senescent MEFs by addition of Cre recombinase to the culture was sufficient to cause cells to re-enter the cell cycle. This highlights the fact that loss of pRb during gestation can result in genetic compensation by the organism.

Screening for new tumor suppressors and oncogenes

Reproducing Bloom's syndrome in mice by removal of one copy of the Bloom's syndrome (*BLM*) gene provides more than just a mouse model for a human condition. As Allan Bradley (The Wellcome Trust Sanger Institute, Hinxton, UK) reported, it also provides a very powerful tool for screening for tumor-suppressor genes and oncogenes. The loss of heterozygosity rates in Bloom's syndrome mice is much higher than in wild-type mice, resulting in a ten-fold increase in sister-chromatid exchange, an increase in mitotic recombination and an increased susceptibility to tumors. Combined with arrays of bacterial artificial chromosomes (BACs) that allow comparative genome hybridization (CGH), it is possible to establish where genomic regions are deleted or amplified in tumors. Another mouse model for screening involves Cre-*loxP*-mediated 'megabase deletion', to generate mice with large intra-chromosomal deletions. Although it is not possible to generate mice with deletions in both copies of a given chromosome (because embryonic lethality arises from the loss of too many genes), heterozygous mice can be examined over time to see if they have increased tumor susceptibility; such an increase might suggest the loss of a tumor suppressor in the deleted region that has also spontaneously been lost or mutated on the other chromosome. Once again, CGH can be used to establish which genes are deleted or amplified on the originally intact chromosome. These screens are in progress (in Bradley's group) and have so far detected known tumor suppressors such as *p53* and adenomatous polyposis coli (*APC*) genes.

Using a comprehensive set of screening approaches, including CGH, cDNA microarray technology and antibody- and peptide-based microarrays, Carlos Cordon-Cardo (Memorial

Sloan-Kettering Cancer Center, New York, USA) identified several proteins that have altered expression in bladder cancer. Of these, elevated cyclin E resulting from gene amplification was identified as a predictive marker for bladder cancer. Altered expression of moesin, a cell-adhesion molecule, and of Her-2, a member of the epidermal growth factor (ErbB/EGF) receptor family of tyrosine kinase signaling molecules, were also associated with poor prognosis and significantly reduced survival in transgenic mouse models.

Signaling pathways

The notion that cell-surface receptors for cytokines and growth factors can transmit both positive and negative signals to cells via the activation of multiple signal-transduction pathways has received much attention in recent years. The importance of this delicate balance between opposing intracellular signals was highlighted in a talk by Matthias Ernst (Ludwig Institute for Cancer Research, Melbourne, Australia). He has been studying the gp130 protein, a common subunit in several cytokine receptors. Signaling by gp130 involves the simultaneous activation of the signaling pathways mediated by the STAT1/3 (signal transducer and activator of transcription) proteins and the phosphatase SHP2. 'Knock-in' gene targeting was used to generate mice with mutant gp130 receptors lacking STAT1/3- or SHP2-signaling capabilities. Loss of gp130-mediated STAT signaling resulted in aberrant repair of mucosal wounds in the gastrointestinal tract, whereas loss of SHP2 signaling from gp130 resulted in the development of gastric adenomas, raising the possibility that gut homeostasis is tightly regulated by opposing SHP2- and STAT-mediated pathways. Biochemical studies supported this hypothesis. Revealingly, the gut phenotypes of the different gp130-mutant mice mirrored those of mice lacking trefoil factors (small cysteine-rich secreted peptides), TFF1 or TFF3, whereas transfection experiments showed that the transcription of *TFF1* and *TFF3* genes is indeed reciprocally regulated by SHP2- and STAT-dependent pathways, respectively.

Immunosurveillance and cancer vaccines

Does immunosurveillance have a role in eliminating tumors? Initiating the discussion on immunosurveillance was Nobel laureate Peter Doherty (St Jude Children's Research Hospital, Memphis, USA). Although his talk focused mainly on CD8⁺ T-cell responses to viruses, we were reminded of the fact that CD8⁺ T-cell-mediated defense does appear to be effective against lymphoma induced by Epstein-Barr virus (EBV). The development of tetramers (tetrameric complexes of the major histocompatibility complex, MHC, plus peptide) has greatly facilitated the detection of antigen-specific T cells. They will allow the question of tumor-specific immunosurveillance to be more carefully addressed because the tetramer interacts only with the T-cell-antigen receptors

that are specific for the tetramer (that is, specific for that MHC-plus-peptide complex).

Examining further the role of tumor immunosurveillance, Mark Smyth (Peter MacCallum Cancer Institute, Melbourne, Australia) has been investigating spontaneous tumor development in aging mice that carry null mutations in a range of genes involved in normal immune-system function. He has found that mice lacking perforin, a key effector molecule in cytotoxic killing by CD8⁺ cells, and also lacking interferon γ (IFN γ) are more susceptible to late-onset adenocarcinoma than are wild-type mice, supporting a role for tumor immunosurveillance in epithelial tissues.

Spontaneous regression of melanomas has been frequently observed and shown to be associated with T-cell infiltrates. On the basis of this observation, Jonathon Cebon (Ludwig Institute, Melbourne, Australia) and colleagues have been trying to develop vaccines including a peptide/peptides in order to generate a T-cell response against melanoma-specific antigens. The vaccine has been combined with Flt-3 ligand in an attempt to enhance antigen presentation by dendritic cells. So far, the frequency of antigen-specific T cells, determined using tetramers after three rounds of immunization, has been very low (less than 1%), while virus-specific T cells have been shown to represent up to 18% of the blood CD8⁺ T-cell population in response to EBV.

Failed attempts to immunize against tumor antigens are thought to result from either a lack of antigen-specific T-cell precursors or ineffective activation and expansion of these cells when challenged with tumor antigen. An approach to overcome this, described by Michael Kershaw (National Institutes of Health, Bethesda, USA) involved the use of dual-specificity T cells. The endogenous T-cell receptor within these cells is specific for allo-antigen, traditionally a very powerful immunogen, whereas specificity for the tumor is conferred by an introduced chimeric receptor comprising the extracellular single-chain form of anti-folate-binding protein (an antigen associated with ovarian cancer) linked to the intracellular domain of Fc γ (the gamma signaling chain of the Fc receptor complex). Although no expansion of the dual-specificity T cells was observed when they were transferred alone into mice bearing tumors, when combined with allogeneic stimulation the cells not only expanded but also resulted in an inhibition of tumor growth. The response was much greater when allogeneic immunization was administered subcutaneously rather than intravenously.

Other markers of cancer

The nine-member Pax family of proteins comprises transcription factors that are normally expressed only during development but are often over-expressed in cancer. Michael Eccles (University of Otago, Dunedin, New Zealand) presented

evidence that expression of one of these proteins, Pax2, confers resistance to apoptotic stimuli.

In discussing other markers of cancer, Jane Visvader (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) argued for a possible link between LMO4, a member of the nuclear LIM-only group of transcriptional regulators containing two tandem LIM zinc-binding domains, and breast tumorigenesis. LMO4 is normally expressed developmentally in the mammary gland and was found to be overexpressed in about 50% of primary human breast cancers. In addition, overexpressing LMO4 in mouse mammary-epithelial cells was shown to inhibit their differentiation. Searching for a mechanism for LMO4 function, Visvader and colleagues identified both the cofactor carboxy-terminal binding protein (CtBP, a protein targeted by adenoviral E1A) and tumor suppressor protein BRCA1 as binding partners for LMO4 and demonstrated repression by LMO4 of BRCA1-mediated transactivation in mammalian cells.

Loss of heterozygosity of the long arm of chromosome 16 is common in breast tumorigenesis. Narrowing the region to 16q24.3, David Callen and colleagues (Bionomics Ltd, Australia) have found a gene, *MTG16*, the expression of which is absent from approximately 50% of breast cancers. Re-introduction of *MTG16* into different breast-cancer-derived cell lines dramatically reduced their proliferative capacity, suggesting that this protein may be a novel tumor suppressor. Karen Boucaut (Queensland Institute of Medical Research, Brisbane, Australia) presented evidence that testisin, a serine proteinase, is a candidate tumor suppressor. Testisin is normally expressed in pre-meiotic spermatocytes but is not expressed in testicular tumors that are thought to arise from germ cells. The absence of testisin expression from the tumors was shown to correlate with extensive methylation of a CpG island (a 200 bp region of high GC content) within the 5'-untranslated region. Furthermore, re-introduction of a membrane-bound form of testisin into the tumor cell line Tera-2 resulted in the formation of smaller tumors in the testes of inoculated immunodeficient SCID mice compared to control-transfected cells.

Metalloproteinases as therapeutic targets

Metalloproteinases are a large family of proteins that mediate degradation of the extracellular matrix. These proteins have been implicated in tumor metastasis and, for this reason, are thought to be good targets for therapeutic intervention. Using xenograft models in nude mice, Mark Waltham (St Vincent's Institute, Melbourne, Australia) has tested the effectiveness of prinomastat, a potent inhibitor of select metalloproteinases, for treatment against breast cancer and breast-to-bone metastasis. A reduction in primary tumor growth and bone metastasis in response to the drug was observed, resulting in significantly reduced

osteolytic bone damage, although metastasis to soft tissue was unaffected. As reported by Lynn Matrisian (Vanderbilt University, Nashville, USA), however, the results of clinical trials of a panel of different metalloproteinase inhibitors for the treatment of a variety of cancers have been generally disappointing. Several factors could have contributed to this, including the finding that in one trial a drug was used although the target metalloproteinase was absent from the tumor, and in another trial a reduction in tumor mass was used as a criterion for effectiveness despite the fact that metalloproteinase inhibitors are cytostatic (preventing cell growth) rather than cytotoxic (damaging to cells). Metalloproteinase inhibitors were not without benefit, however, and in one trial improved the outcome of patients who had received prior chemotherapy. The need to treat patients earlier and to refine the treatments for the specific tumors being targeted was highlighted.

As we try to unravel the mysteries of tumorigenesis, it is becoming increasingly clear that for every type of cancer - and there are many - deregulation and/or mutation of a specific subset of genes is likely to be involved. The affected genes may include oncogenes that become activated or amplified leading to enhanced cell growth, or tumor suppressors that are lost and fail to oppose it. For every tumor we need to know the precise molecular basis of disease: which genes have been mutated or silenced, and what proteins are over-represented or absent? The importance of 'knowing your tumor' so that individually tailored tumor therapy can be put into place was an emerging theme at the Lorne Cancer Conference and one that seems likely to have an impact in the clinic in the not too distant future.

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