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PROTEIN KINASES AND PROTEIN PHOSPHATASES: MINING THE KINOME AND PHOSPHATOME FOR NOVEL DISEASE GENES.

Protein kinases and protein phosphatases are two classes of enzymes that act as molecular switches to control almost every biological process in the body. When the genes for these enzymes are mutated they can effectively leave the switches permanently on or permanently off. This can result in developmental defects, degenerative disorders and cancer. Examples of diseases associated with these enzymes include diabetes, deafness, epilepsy, mental retardation, muscular dystrophy, motor neuropathy, Parkinson's disease, and multiple forms of cancer such as melanoma, colon cancer, colorectal cancer, leukaemia, and prostate cancer. In mammals, there are approximately 690 protein kinases and protein phosphatases, of which 290 are associated with disease in humans or mice. Many of the remaining genes are poorly characterised, and given the number already known to be involved in disease it makes study of these genes important. Using a combination of "bioinformatics" (computational biology) and traditional bench experiments we have developed an online encyclopaedia of all mammalian protein kinases and phosphatases that details where the enzyme is expressed in the body, which part of the cell it targets to, the pathways the enzyme is involved in, the proteins it works upon, the structure of the gene and whether the enzyme is associated with a disease in mice or humans. By reviewing the structure of the genes we have also shown that through a process called alternative splicing at least 75% of these genes produce alternative forms which are likely to have important implications in biology and disease. This talk will give an overview of these enzymes, and how the study of the entire set including the alternative forms is important. Finally we invite the community to view the database online at (http://phosphoreg.imb.uq.edu.au/home.shtml).

Protein kinases and protein phosphatases: mining the kinome and phosphatome for novel disease genes.

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Protein kinases and protein phosphatases are key regulators of cell proliferation, cell signaling and development. Disruption of the genes encoding these enzymes is associated with many diseases including diabetes, deafness, epilepsy, cardiomyopathy, mental retardation, myotonic dystrophy, motor neuropathy, parkinsons disease, severe combined immunodeficiency and multiple forms of cancer such as melanoma, colon cancer, colorectal cancer, leukaemia, and prostate cancer. Of the 690 kinases and phosphatases common to both human and mouse, 290 have recorded mutant phenotypes, 96 of which have tumour associations. By comparing both mouse and human data we identify 142 mouse phenotypes yet to be associated with any human disease. Furthermore there remains a further 400 genes for which phenotypes have not yet been determined. These numbers suggest greater characterization of these loci is likely to reveal further disease associations. To systematically characterize the kinase and phosphatase complement of mammals, we have used a combination of bioinformatics and traditional bench experiments to functionally annotate the loci and the transcripts generated from each. This has yielded two database tools for the study of mammalian protein kinases and phosphatases, the first PhosphoregDB (http://phosphoreg.imb.uq.edu.au/home.shtml), provides summary information for each locus, the pathways the protein is involved in, the proteins it interacts with, known substrates, sub-cellular localization, classification and tissue distribution. The second database VariantDB (http://variant.imb.uq.edu.au) provides a genomic view of every kinase and phosphatase gene and shows that 75% of these loci generate alternative transcript isoforms. Together these tools provide an integrated framework for further studies examining 1) the expression and mutation status of these transcripts in normal and disease states, and 2) the effect of knockdown and over-expression of these genes on cell proliferation.

Alberto Pinzon-Charry

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ABNORMALITIES IN DENDRITIC CELLS IN PATIENTS WITH CANCER

Effective responses against tumours rely on the coordinated action of the immune system. The immune system is a complex and effective network of specialised cells, organs and factors (cytokines) capable of identifying and removing cancer cells. Amongst immune cells, dendritic cells (DC) are extremely important because they have the unique capacity to initiate and coordinate the different arms of the immune system against cancer. Therefore, in spite of being very rare white blood cells, there is much interest in exploiting DC to target and destroy cancer cells. This type of anti-cancer therapy (DC immune therapy) involves the alteration of the body's own DC in vitro (test tube), for subsequent administration to the patient to produce potent anti-tumour effects. We studied one hundred and fifty patients with cancer (breast, prostate and brain) to determine if tumours affected their DC's ultimately aiming to find ways to improve DC as an anti-cancer therapy. We found that a great number of DC in patients with cancer but not in healthy volunteers were dying in blood, indicating that tumours were inducing death of these potent immune cells. We then set out to identify factors that would prevent death of DC. One of the factors tested, CD40L, improved DC function and protected DC from death. Together with dying DC, we also found a great number of immature (inefficient) DC in blood of cancer patients. Interestingly, the more advanced the cancer, the greater the number of immature DC in blood suggesting that tumors were impacting on the body's capacity to generate efficient (mature) DC. In contrast to mature DC found on most healthy volunteers, these immature DC abundant in patients with cancer were found to fail in generating anti-cancer responses. More importantly, CD40L, the same factor that protected DC from death, improved the function of mature and induced the maturation of immature DC boosting their capacity to generate anti-tumor responses. Our data is relevant because it sheds some light onto an important mechanism underlying the failure of the body's immune system to respond to tumors in cancer patients. Our data may prove to be crucial in improving the efficacy of immunotherapy for different types of cancer.

Multiple abnormalities in blood dendritic cell compartment in patients with solid tumors

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Dendritic cells (DC) are key antigen-presenting cells (APC) that play an essential role in initiating and directing cellular and humoral immunity including anti-tumor responses. Due to their critical role in cancer, the design of DC vaccines to prevent the recurrence or achieve the regression of already existing tumors has been a major goal of immunotherapy. Blood DCs have been proposed for such immunotherapy protocols because they offer the theoretical advantage of being in their natural state of differentiation and are capable of orchestrating immune responses in a more physiological manner. We have thoroughly assessed the circulating DC compartment (Lin-HLA-DR+ cells) in 149 patients with breast cancer, prostate cancer and malignant glioma performing phenotypic, quantitative and functional analyses at various stage of disease. We document the presence of a significantly higher proportion of apoptotic blood DC in patients with cancer compared to healthy volunteers confirming the role of tumor products in this phenomenon. Indeed, supernatants derived from cancer lines but not control cultures induce DC apoptosis through down-regulation of the anti-apoptotic molecule Bcl-2. To identify factors that would prevent DC apoptosis and thus harness DC's immunotherapeutic potential we sought to test a range of clinically available DC maturation stimuli. We found that most stimuli (inflammatory cytokines and double stranded RNA) induced maturation but failed to protect DC from apoptosis. In contrast, CD40 stimulation induced vigorous maturation and protected DC from apoptosis through sustained expression of Bcl-2 further supporting this approach for immunotherapy. Our observation of a higher fraction of blood DC undergoing apoptosis was also paralleled by the paucity of conventional DC and the accumulation of a previously undefined population of immature cells in blood of cancer patients. As with apoptotic DC, increased numbers of immature cells in blood correlated with disease status. Indeed, patients with metastatic cancer showed a larger number of immature cells in circulation than patients with local disease. Immature cells exhibited limited capacity to activate T-cells and induced a deleterious type of activation profile in immune effectors when compared to DC. However, CD40 stimulation (but no other stimuli i.e., inflammatory cytokines, polyI:C or lipopolysaccharide) was capable of inducing both a vigorous activation of DC and robust maturation of all immature cells, in turn generating more efficient immune responses. Our results demonstrate evidence for the direct immunosuppressive effect of tumors on DC and suggest that impairment of blood DC correlating with accumulation of immature cells with poor immunologic function associates with disease progression.

Natalie J Colson

Genomics Research Centre and Institute of Environmental Science and Research

GENETIC VARIANTS IN HORMONAL AND VASCULAR GENES PLAY A ROLE IN MIGRAINE

Migraine is a painful and debilitating condition affecting up to 12% of the population. Apart from the significant toll in human suffering, migraine also imposes a serious economic burden on society due to the associated costs of medical care, treatment, and lost productivity. Diagnosis is problematic and misdiagnosis can lead to failure to appropriately treat the disorder. Furthermore, treatment is not always successful. The causes of migraine remain unclear although family and twin studies show that genes certainly play a role. Thus our research aimed to identify genes involved in migraine susceptibility.

We studied genes involved in hormonal and vascular pathways as hormones are a well known migraine trigger in women and blood vessel changes have been reported both prior to and during a migraine attack. These genes were investigated in 550 migraine sufferers and 550 individuals who have never suffered migraine to determine if differences in these genes occurred between the two groups. Our results showed that for two hormonal and one vascular gene, specific gene variations occurred at a much higher frequency in the migraine group. Thus individuals who possessed these specific gene variations were at a higher risk of suffering migraine than those who did not. When we analysed our results along with previously determined vascular gene susceptibility variants we found that individuals who carried specific hormonal or vascular gene variations were 4 times more likely to suffer migraine. Individuals who carried both the hormonal and vascular gene variations were 7 times more likely to suffer migraine.

This is the first time that a role for hormone related genes has been shown in migraine as well as further evidence for a role of vascular genes in migraine. We have also provided interesting preliminary results in gene risk profiling with the potential to lead to more accurate and reliable diagnostic options and the development of personalized treatment.

Genetic variants in hormonal and vascular genes play a role in migraine

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Migraine is a chronic and debilitating neurological disorder affecting up to 12% of the population. Apart from the significant toll in human suffering, migraine also imposes a serious economic burden on society due to the associated costs of medical care, treatment, and lost productivity. Diagnosis is problematic and misdiagnosis can lead to incorrect therapy or failure to appropriately treat the disorder. In addition, the efficacy of current treatment options is variable. While the pathophysiology of migraine is not fully understood, it is clear that there is a significant genetic component to the disorder. Neurotransmitter related pathways have been the main focus of studies investigating the molecular mechanisms of the disorder. However vascular and hormonal disturbances also occur in migraineurs, as highlighted by alterations in cerebral blood flow and hormonal triggers of migraine. Hence factors affecting these functions may play a causative role in the disorder. This study investigated hormonal and vascular gene variants as potential migraine susceptibility genes. The hormonal variants investigated were the estrogen receptor 1 (ESR1) 1 gene G594A polymorphism, PvuII polymorphism, and C325G polymorphism as well as the progesterone receptor (PGR) gene PROGINS insert and androgen receptor (AR) gene CAG repeat. The vascular variants investigated were the methylenetetrahydrofolate reductase (MTHFR) gene A1298C polymorphism and the methionine synthase reductase (MSR) gene A66G variant. The ESR1 G594A variant showed significant differences between the migraine and control groups (P=0.008). These significant results were followed up in a second independent population (P=4x10- 5). Analysis of the PGR variant also showed significant differences between the migraine and control groups (P = 0.039). This is the first time that these ESR1 and PGR variants have been demonstrated to play a role in migraine. Furthermore, it has been shown in two large matched independent populations. Analysis of the MTHFR variant also suggested a role in migraine (P = 0.015). We created hormonal and vascular risk profiles by coding individuals according to details of susceptibility variants for ESR1 594A, PGR, MTHFR 1298C, and previously determined MTHFR 677T and angiotensin converting enzyme (ACE) gene risk variants. Results of this analysis revealed that individuals who possess a complete vascular or hormonal risk profile are 4.4 times more likely to suffer migraine (P =7x10-4). Individuals who possess 2 complete risk profiles are ~7 times more likely to suffer migraine (P = 0.012). This is the first time that a role for hormone related genes has been shown in migraine. We have also provided additional evidence for the role of MTHFR in migraine. Furthermore, we have provided interesting preliminary results in genetic risk profiling with the potential to lead to more accurate and reliable diagnostic options and the development of better therapeutics.

Jason G. Kay

Institute for Molecular Bioscience, University of Queensland

CHOLESTEROL IN CUPS IS CENTRAL FOR CYTOKINE SECRETION

Macrophages are white blood cells that function at the frontline of immunity. We have studied the pathway for releasing a potent cytokine that regulates the immune response, TNF, from macrophages. We have described an efficient pathway in macrophages that releases TNF and devours microbes at the same time. To do this, TNF delivery occurs at a special site on the cell surface having a concentration of membrane fusion proteins in cholesterol rich domains. Cholesterol is essential for TNF release and for the ingestion of microbes and now offers new avenues for regulating TNF secretion as a major factor in inflammatory disease.

Cholesterol in cups are central for cytokine secretion

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Tumour necrosis factor alpha (TNF) is a potent, multipurpose cytokine with the critical task of initiating an immune response when released by activated macrophages at sites of inflammation or microbial infection. In such situations, TNF must be produced in abundance and secreted quickly by macrophages. The intracellular pathways and trafficking molecules responsible for TNF release are just beginning to be understood, based largely on studies from our lab. My project has focused on the later stages in TNF secretion, where it is delivered to the cell surface as a full length protein, from where it is cleaved and released by a metalloprotease (TACE). A major finding from my work was to elucidate the pathway for TNF delivery to the surface using cell and molecular biology approaches. This showed unexpectedly that TNF was delivered to the phagocytic cup, along with internal membrane that makes the cup to engulf microbes. By imaging GFP-tagged TNF in live macrophages, I was able to capture the joint delivery of TNF to the cell surface and ingestion of yeast. I was also able to confirm that proteins responsible for TNF delivery and release, including membrane fusion proteins and TACE, are also clustered at this site. The phagocytic cup was thus revealed as the site for TNF release from cells via a pathway that ensues rapid and efficient delivery (Murray et al, Science, 2005). What helps make this area of the cell membrane such an abundant release site? The phagocytic cup contains an increased concentration of cholesterol enriched lipid raftdomains. By biochemical separation of these raft domains I was able to show that the membrane fusion machinery for delivering TNF to the cell surface is enriched in the cholesterol-lipid rafts; moreover, the key fusion protein at this site, syntaxin 4, is recruited in a cholesterol-dependent manner when the macrophages are activated to start producing cytokine. Confocal imaging confirmed that syntaxin 4 localised at the raft domains is the precise site where TNF is delivered to the cell surface, from where it can be released. Thus when macrophages are activated by contact with a microbe, lipid rafts are deployed to form exit sites at the cell surface, in readiness for the rapid secretion of the early response inflammatory cytokine TNF. Indeed, I found that since these rafts are in the phagocytic cups, they are also necessary for efficient phagocytosis (Kay et al, JBC,2006). The results from the current studies show how TNF is targeted to cell surface sites in forming phagocytic cups, so that TNF can be rapidly released prior to phagocytosis of microbes. The exit sites contain increased amounts of cholesterol, which is required to form membrane fusion sites for cytokine secretion. Based on these new findings, future studies will pursue cholesterolreducing drugs, such as statins, as novel therapeutic strategies for controlling TNF secretion and combating the excess cytokine secretion underlying many chronic inflammatory diseases

Leisl Packer

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A FUNCTIONAL GENETICS APPROACH TO UNDERSTANDING MELANOMA DEVELOPMENT

This research aims to better understand the genetic background of melanoma, so that we can understand which genes are involved in melanoma development. We are looking at thousands of genes to identify which genes are "misbehaving" in melanoma cells. We want to find out exactly what functions these genes perform in normal and malignant cells as this will enable us to design new therapies to "fix" the genes that are changed or "misbehaving" in the melanoma cells. Thus our overall aim is to eventually find new treatments to slow or stop the progression of melanoma in the body.

A functional genetics approach to understanding melanoma development

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Malignant melanoma is the most deadly form of skin cancer and the incidence is rising faster than any other cancer. There are currently no effective therapeutic strategies for treating metastatic melanoma. Several genes have been shown to be involved in melanoma susceptibility and progression, but little is known about their exact contribution to tumour development and none have proven to be effective targets in treated this disease. Thus, better prognostic markers and therapeutic targets for melanoma are urgently needed. We have used molecular and cellular profiling to sub-classify melanomas on the basis of genotype and phenotype with the aim of identifying novel genes involved in melanoma development and progression. Gene expression profiling of 63 melanoma cell lines was carried out using Affymetrix whole-genome microarrays. To identify potential downstream effectors of some key tumour suppressors and oncogenes involved in melanoma, the melanoma cell lines were sequenced to determine which cell lines carried mutant or wild-type copies of these genes. Microarray data was analysed in conjunction with the mutation status of these melanoma-susceptibility genes to identify genes whose expression correlates with a particular genotype. We have thus far published the gene expression profiles associated with BRAF, NRAS and PTEN genotypes in melanoma. A small subset of genes differentially expressed between PTEN mutant and wild-type cell lines have undergone functional analysis to confirm these genes are modulated by PTEN. The main finding from this study was the increased expression of osteopontin when PTEN is lost. This inverse correlation was observed at the mRNA and protein level in our melanoma cell lines and human tissues of a melanoma tissue microarray. Osteopontin has been shown to promote tumourigenesis in many cancer types and our results suggest that osteopontin may be involved in melanoma development when PTEN is inactivated. Analysis of the gene expression profiles associated with the p14ARF tumour suppressor is currently underway. RNAi and induction of p14ARF in melanoma cell lines are being performed to determine the effect on potential downstream effectors selected from the microarray analysis. By characterizing genes modulated by mutations in key melanoma-susceptibility genes we hope to identify novel therapeutic targets in patients harbouring these specific mutations. The final aim of this research is to identify genes involved in melanoma invasion and progression. To achieve this aim, the melanoma cell lines were phenotypically characterized using a variety of cellular assays including: growth rate, strength of adhesion, invasive properties such as cell motility, their ability to degrade extracellular matrix and basement membranes and their ability to form tubular structures which are associated with poor prognosis in vivo. We have identified 203 genes associated with invasive ability using the microarray data. A small number of genes from this list will be chosen for future functional tests to confirm their role in melanoma progression, with the hope that some may be novel prognostic markers or targets for therapeutic intervention in metastatic melanoma patients.

Penney Jeffery

Ghrelin Research Group

THE ROLES OF GHRELIN AND GHRELIN VARIANTS IN CANCER

Ghrelin is a newly discovered hormone that is a potent stimulator of appetite and that possesses a wide range of activities in the body. Recently, a number of important variant forms of ghrelin have been identified, including a truncated ghrelin peptide that was first isolated in our laboratory. This novel ghrelin peptide is also widely expressed in the mouse, indicating that it is a highly conserved peptide and may have an important role within the ghrelin axis. We have demonstrated that ghrelin and this unique ghrelin variant are highly expressed in hormone-dependent cancers including prostate, breast and ovarian cancers and that ghrelin exerts a growth-stimulating effect on cell lines derived from prostate and breast cancer. Hormone-dependent cancer has a significant impact upon morbidity and mortality rates in the Western world. There is now considerable evidence that up-regulated growth factor activity promotes an aggressive phenotype that is resistant to traditional hormonal therapies and is associated with a poor prognosis. This highlights the need for adjunctive therapies that can prevent, or overcome, hormone-refractory disease. This study supports a role for ghrelin as an important growth factor for hormone-dependent cancer and therefore the ghrelin axis represents a novel target for the treatment of such cancers in the future. We have also identified several potential diagnostic markers for prostate, breast and ovarian cancer within the ghrelin axis.

The role of the ghrelin axis in hormone-dependent cancer and characterisation of a novel exon 3-deleted preproghrelin isoform and its murine homologue

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Ghrelin is a 28 amino acid peptide hormone that has an extensive range of physiological effects, including stimulation of growth hormone (GH) release and appetite regulation. The cognate receptor for ghrelin is the growth hormone secretagogue receptor (GHS-R), a G protein-coupled receptor with two documented isoforms, the functional GHS-R type 1a and the C-terminally truncated GHS-R type 1b. In our laboratory, we have identified a novel exon 3-deleted preproghrelin variant that retains sequence for the mature ghrelin hormone and also encodes a novel C-terminal peptide (designated as C-terminal 3 peptide) which has a murine counterpart, exon 4-deleted preproghrelin, indicating that it is highly conserved1. There is now substantial evidence to suggest that the ghrelin axis, encompassing ghrelin, several ghrelin variants and both forms of the GHS-R, is implicated in hormone-dependent cancer. Ghrelin and exon 3-deleted preproghrelin are highly expressed in prostate cancer tissues compared to expression levels in normal prostate glands2. Similarly, breast carcinoma specimens display greater immunoreactivity for ghrelin and exon 3-deleted preproghrelin than normal breast tissues. Expression of the exon 3-deleted preproghrelin mRNA isoform is upregulated in the oestrogen-independent, highly malignant MDA-MB-435 breast cancer cell line compared to the non-tumourigenic MCF-10A breast epithelial cell line, suggesting that augmented transcription of the isoform is associated with an increased malignant potential in breast cancer3. This result is also reflected in ovarian cancer cells lines, with expression of full-length and exon 3-deleted preproghrelin transcripts upregulated in ovarian cancer cell lines compared to immortalized ovarian surface epithelial cells. These studies have also been the first to demonstrate that ghrelin may have an important role in cell proliferation in breast and prostate cancer via rapid activation the ERK 1/2 mitogen-activated kinase (MAPK) pathways. Prostate cancer cells secrete mature ghrelin in vitro, and may therefore stimulate MAPK pathways in an autocrine manner. In summary, this study has provided new and compelling evidence that supports a role for the ghrelin axis in hormone-dependent cancer and specifically in prostate and breast cancer. We have identified several potential diagnostic markers for prostate and breast cancer within the ghrelin axis. Ghrelin stimulates proliferation of hormone-dependent cancer cells and therefore the ghrelin axis represents a novel target for the treatment of such cancers. This project has also provided a basis for future in vivo work in mouse models, which will potentially aid in the development of new adjunctive therapies for prostate and breast cancer.

Ian M. Mackay

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NEWLY IDENTIFIED VIRUSES IN ACUTE RESPIRATORY TRACT INFECTIONS OF CHILDREN

Respiratory illnesses like the common cold and serious chest colds usually arise after infection by respiratory viruses and mostly occur in children. There are also few studies of how viruses that cause head and chest colds change themselves over time and whether some variants are more likely to be found in patients with a particular illness. The aim of our studies was to better understand these viruses. Respiratory viruses are often present in children with pneumonia, are the most frequently identified infectious agent found in asthma exacerbations and are the most common reason for prescribing antibiotics (more so even than bacterial infections) which are of no use on viruses. To study these viruses in detail we first had to develop tests capable of detecting very small amounts of viral genetic material and then used the tests to screen specimens from ill children visiting Queensland hospitals.

In one study we improved the rate of virus detection by four-fold and found that the most commonly detected viruses were picornaviruses. We also found that these viruses were the sole pathogen detected in more than a third of lower respiratory tract infections, which are considerably more serious and expensive illnesses to manage than common colds. We completed a series of additional studies of four recently described respiratory viruses resulting in the identification and characterisation of each for the first time in Australia. Our studies found that some viruses were detected alongside up to three other viruses in the same specimen. Most recently we have found a new picornavirus-like virus in young children.

Our research findings are an important part of global respiratory virus studies. Not only are "new" viruses being identified, but modern detection and characterisation of viruses is helping to show that previous studies assigning some viruses to minor roles in respiratory illness were misleading. We are involved in the important work of building the capability to detect all common human respiratory viruses in children with the long term goals of improving respiratory virus research and reducing the spread and economic impact of viral respiratory illness and mortality especially during peak respiratory seasons, outbreaks and epidemics. Better understanding will help to improve the quality of patient care, minimise the spread of illness and provide the knowledge required to focus future antiviral drug and vaccine development.

Newly identified viruses in acute respiratory tract infections of children

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Studies at the Queensland paediatric infectious diseases (Q pid) laboratory indicate that up to 85% of specimens collected from children with a clinically identified acute respiratory tract infection (ARTI) are not provided a laboratory diagnosis. Studies worldwide describe equally poor detection rates. Because ARTIs are the most frequent infection of children and viruses are the most common microbe detected in ARTIs, our studies have sought to establish the extent of involvement of under-characterised, recently identified and previously unknown viruses in paediatric ARTI. These viruses constitute the most common reason for prescribing antibiotics (more so even than bacterial infections) which would be ineffective, are often present in children with bronchiolitis and are the most frequently identified pathogens found in asthma exacerbations, making them a significant drain on healthcare resources at many levels.

In one study we improved the rate of virus diagnoses by four-fold and found that the most commonly detected viruses were picornaviruses. We also showed that these viruses were the sole microbe detected in 38% of lower respiratory tract infections (LRTI) which are considerably more serious and expensive illnesses to manage than common colds. Our studies found that in 18% of specimens a virus, particularly a DNA virus, was co-detected with up to three other viruses. Further studies have led to the preliminary identification of a previously undescribed picornavirus-like virus (PLV) which predominantly infects children under 5yrs of age, throughout the year and is a significant respiratory pathogen. The prototype virus, PLV-1 was found in a child with rhinorrhoea, cough, otitis media and wheeze. Chest X-ray results were consistent with a non-specific viral LRTI which required hospital admission. PLV-1 is prevalent in 2% of specimens tested to date. PLV-1 exhibits characteristics of members of the family Picornaviridae and further characterisation is ongoing.

Our findings are an integral part of a global renaissance in respiratory virus discovery, the like of which has not been seen since the 1960s. Not only are "new" viruses being identified, but molecular detection and characterisation of viruses is helping to show that previous studies assigning some viruses to minor roles in respiratory illness were misleading. Since clinical markers alone are inadequate better understanding of the role for respiratory viruses in paediatric hospital populations will help to improve the quality of patient care, minimise the spread of illness and provide the knowledge required to focus future antiviral drug and vaccine development projects.

Jos Malda

Tissue Regeneration and Repair Program, Institute of Health and Biomedical Innovation, Queensland University of Technology

HYPERBARIC OXYGEN IN WOUND HEALING: FRIEND OR FOE?

Wound healing is a complex and highly orchestrated process involving phases of inflammation, proliferation, and remodelling. Oxygen is one of the critical nutrients during wound healing and plays a central role in the repair. The requirement for oxygen during the process of wound healing provides the underlying rationale for hyperbaric oxygen (HBO) therapy. HBO involves the administration of 100% oxygen under pressure, which elevates oxygen levels in the body significantly. Though often used, the clinical application of HBO therapy to assist healing of non-healing wounds remains a subject of great debate. Therefore, we sought to determine if repetitive HBO treatments would influence the formation of skin using laboratory human skin equivalent (HSE) model. Models were exposed to daily HBO treatments (90 min, 100% oxygen at 2.4ATA) using our custom-designed laboratory-scale hyperbaric chamber.

The results revealed that the outer protective layer of the skin (epidermis) was significantly thicker at both day 3 and day 5 in HBO-treated samples compared to the non-treated controls. In addition, characterization of the newly-formed tissue confirmed the earlier formation of an epidermis within the HBO-treated constructs.

Taken together these results demonstrate, for the first time, that HBO stimulates the formation of an epidermis using a relevant laboratory-scale model based on human cells. These findings will further facilitate the elucidation of mechanisms underlying the HBO therapy. Clearly, any wound care intervention that can prevent even a small fraction of wounds from progressing to the stage where on-going care is required will have a significant, favourable impact on quality of life, as well as a significant economic impact.

Hyperbaric oxygen in wound healing: friend or foe

Jos Malda, Evette Kairuz, Rebacca Dawson and Zee Upton

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Wound healing is a complex and highly orchestrated process involving phases of inflammation, proliferation, and remodelling. Oxygen is one of the critical nutrients during wound healing and plays a central role in the reparative process. The requirement for oxygen during the process of wound healing provides the underlying rationale for hyperbaric oxygen (HBO) therapy. Though often used, the clinical application of HBO therapy to assist healing of chronic wounds remains a subject of great debate. Moreover, current in vitro data on the effect of HBO is limited and inconclusive and the effects of HBO on keratinocytes and re-epithelialisation are even less well understood. Therefore, we sought to determine if repetitive HBO treatments would influence the reconstruction of an epidermis using an ex vivo human skin equivalent (HSE) model.

Human skin equivalents were constructed from de-epidermised skin as previously described1,2 and cultured at the air-liquid interface for up to 5 days. HBO treatments (90 min, 100% oxygen at 2.4ATA) were given daily using our custom-designed laboratory-scale hyperbaric chamber and 3 independent replicates of the experiment were performed.

Image analysis of hematoxylin and eosin stained cross-sections of the HSE models revealed that the reconstructed epidermal layer in HBO-treated samples was significantly thicker at both day 3 and day 5 compared to the non-treated controls.

In addition, immunohistological characterization of the HSEs using various epidermal markers, including P63, cytokeratins 1/10/11, 6 and 14 and collagen type IV, confirmed the earlier onset of epidermal differentiation within the HBO-treated constructs (results not shown). Moreover, after 3 days of culture, the populated surface area (lateral migration) was significantly greater for the HBO-treated samples compared to the controls (mean $\pm$ SD, 0.58

$\pm$ 0.06 cm2 for HBO, and 0.46 $\pm$ 0.03 cm2 for the control). Although a similar difference was observed between the HBO-treated and non-treated samples after 5 days, this was not significant.

Taken together these results demonstrate, for the first time, that HBO stimulates early onset of the re-epithelialisation, epidermal maturation and stratification using a relevant ex-vivo human model. These findings will further facilitate the elucidation of mechanisms underlying the HBO therapy. Clearly, any wound care intervention that can prevent even a small fraction of wounds from progressing to the stage where on-going care is required will have a significant, favourable impact on quality of life as well as a significant economic impact.

Dr Stuart Macgregor

Queensland Institute of Medical Research

A NOVEL METHOD FOR THE ANALYSIS OF POOLED DNA SAMPLES ON HIGH DENSITY ARRAYS

Genetic studies over the last 20 years have increased our understanding of the underlying biology for a range of diseases. Previously, successful studies utilised family-based samples (using sparse sets of genetic markers). New technologies now allow denser sets of markers, facilitating the use of a powerful population-based study design. However, this is often prohibitively expensive. The cost can be reduced by using DNA "pooling". We have shown that advanced statistical methods are required for analysis and interpretation of pooling data. Coupled with our new statistical method, DNA pooling offers up to 40-fold reduction in cost compared with alternatives.

We have applied our novel statistical method to data on endometriosis. Endometriosis affects up to ten percent of women and is associated with pelvic pain and infertility. By applying pooling techniques to large samples of endometriosis cases and controls we have been able to identify genetic variants contributing to endometriosis susceptibility. Knowledge of the relevant genetic variants will enable us to define pathways leading to disease.

A novel method for the analysis of pooled DNA samples on high density arrays

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Many complex traits and common diseases have a substantial genetic component. Major efforts have been made to identify the loci underlying this component. Identification of such loci is likely to lead to greatly improved understanding of the biochemical and developmental pathways involved in each disease. Due to the limitations of genotyping technology, recent progress has been largely based on mapping genes using a two stage strategy in which an initial genetic linkage analysis was followed by a small scale genetic association analysis. Recently, large scale genotyping has become tenable, making genome-wide association (GWA) analysis a very hot topic. It is now clear that GWA is set to become one of the primary tools for the identification of loci contributing to susceptibility to complex common human disease. GWA will require genotyping hundreds of thousands of single nucleotide polymorphisms (SNPs) across the genome, at very substantial cost. Individually genotyping samples of interest is prohibitively expensive, with genome scans of suitable size (hundreds of cases/controls, hundreds of thousands of markers) typically costing well over $1 million. Alternative approaches are therefore required to reduce the genotyping cost. The most promising approach for reducing cost is DNA pooling. In this, instead of individually genotyping every person in the sample, the sample is genotyped in pools of individuals. A simple test for genetic association can be constructed by comparing the allele frequencies in the case pool and in the control pool. However, naive tests derived from DNA pool allele frequency estimates have undesirable statistical properties and lead to dramatic increases in false positives. A more appropriate test can be derived by recognising that DNA pools yield estimated allele counts rather than observed counts. Essentially, the additional error variance generated by pooling must be taken into account.We propose a novel statistical method for analysis of large scale (array based) pooling data which utilizes the information available across multiple SNPs to estimate the errors inherent in pooling. By utilizing the information from multiple SNPs we are able to estimate the variance associated with pooling. This allows us to construct a statistical test for association with desirable properties. Moreover, since array data will typically have a regular structure (in terms of multiple measurements per SNP on the array), simple tests (such as t-tests) which ignore this structure will be unsatisfactory. We propose the use of general linear model based tests which take into account the structure of the array data. Since the error variance associated with pooling is estimated across SNPs, the need for replication of pools is minimized, thereby decreasing cost. The method does not require prior information on the value of k (a measure of the extent of unequal amplification/hybridization of alleles) and hence avoids the need for expensive individual genotyping of heterozygotes for every SNP of interest. Therefore our method easily scales up to arrays with hundreds of thousands to millions of SNPs. Our new method is applied to data on a set of 384 cases and controls from a study on endometriosis typed with the Affymetrix Genechip© HindIII array. For a subset of this data there were accurate measures of k available. We show that assuming k=1 has a negligible effect upon the results. We only had access to a very small number of arrays (6 in total). Nonetheless, our method extracted 1/3 of the information (in terms of equivalent sample size) available with individual genotyping (requiring 768 arrays). That is, with our novel statistical approach, pooling offers a >40 fold reduction in genotyping cost. With a larger number of arrays a greater proportion of the total information can be extracted. For example, with 20 arrays (10 for cases, 10 for controls), over half of the information could be extracted from this sample.

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GENOME-WIDE SIGNIFICANT LINKAGE TO MIGRAINOUS HEADACHE

Typical migraine is a frequent, debilitating and painful disorder that normally affects people during their most productive years (25% of females and 7.5% of males). The World Health Organization recently identified migraine among the world's top 20 leading causes of disability, with an impact that extends far beyond the suffering individual, to the family and community. Although migraine is highly prevalent in our society, its aetiology remains relatively obscure and there are no laboratory based diagnostic tests that identify those who suffer from the disorder.

Twin studies indicate that migraine has a significant genetic component, with heritability estimates of 33-65%. Therefore, in an effort to identify the molecular mechanisms underlying the disorder, we have been looking for genomic regions co-inherited (linked) with migraine.

The resulting genome-wide linkage scan involving 756 Australian migraine families found significant evidence for the presence of a novel migraine gene on chromosome 5q21 and highly suggestive evidence for a gene on chromosome 10q22. Importantly, we recently replicated linkage to the 5q21 and 10q22 regions in an independent collection of 147 Australian migraine families. Moreover, analysis of migraine trait components (i.e., individual symptoms) in these families produced significant evidence for linkage to the 10q22 region. Consequently, these regions hold great promise for identifying migraine susceptibility genes in our population.

Using a bioinformatics computer-assisted search of public databases we have ranked the potential candidature of the 177 genes within the 5q21 and 10q22 implicated regions.

We are currently designing a detailed screen of the 21 highest priority candidate genes in our two strongly implicated regions, to identify genes underlying common migraine susceptibility. The identification of these genes will provide clues to the further elucidation of the complex molecular pathways of migraine and, finally, will help in the development of diagnostic tests and rational treatment strategies.

Genome-wide significant linkage to migrainous headache

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Typical migraine is a frequent, debilitating and painful disorder that normally affects people during their most productive years (25% of females and 7.5% of males). The World Health Organization recently identified migraine among the world's top 20 leading causes of disability, with an impact that extends far beyond the suffering individual, to the family and community. Although migraine is highly prevalent in our society, its aetiology remains relatively obscure and there are no laboratory based diagnostic tests that identify those who suffer from the disorder.

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Migraine is a symptom complex with variable symptom profiles and individuals presenting with dissimilar symptoms can equally satisfy the same diagnosis. Therefore, to more accurately diagnose migrainous headache, we utilised an empirical clustering approach called "latent class analysis" (LCA)—a statistical method, closely analogous to cluster analysis, for finding subtypes of related individuals (latent classes) from multivariate categorical data—to identify subgroups of people with migrainous headache in a community sample of 12,245 Australian twins (60% female), drawn from two cohorts of individuals aged 23–90 years who completed an interview based on International Headache Society criteria.

We recently reported [Nyholt et al. Am J Hum Genet. 2005 77(3):500-512] results from genome-wide linkage analyses involving 756 twin families containing a total of 790 independent sibling pairs [130 affected concordant (A-A), 324 discordant (A-U) and 336 unaffected concordant (U-U) for migrainous headache]. Quantitative-trait linkage analysis produced evidence of significant linkage on chromosome 5q21, highly suggestive evidence for a gene on chromosome 10q22 and suggestive linkage on chromosomes 8 and 13. In addition, we replicated previously reported typical-migraine susceptibility loci on chromosomes 6p12.2-p21.1 and 1q21-q23, the latter being within 3 cM of the rare autosomal dominant familial hemiplegic migraine (FHM) gene (ATP1A2); a finding which potentially implicates ATP1A2 in familial typical migraine for the first time. Linkage analyses of individual migraine symptoms for our six most interesting chromosomes provided tantalizing hints of the phenotypic and genetic complexity of migraine.

Most recently, we report the results of genome-wide linkage analyses involving 147 additional migraine families containing a total of 368 affected and 144 unaffected children, giving a total of 360 equivalent independent sibling pairs (216 A-A, 97 A-U and 47 U-U), where we have replicated linkage to the two most significant regions with p-values of 0.01, within 9 cM and 2 cM of the original 5q and 10q peak linkage (LOD) scores, respectively. Moreover, analysis of migraine trait components (i.e., individual symptoms) in these families produced significant evidence for linkage (LOD=4.23) to the 10q region. Consequently, the 5q and 10q regions hold great promise for identifying migraine susceptibility genes in our Australian sample.

The 95% confidence interval (CI) for the 5q and 10q loci have been examined in detail by use of GeneSniffer, a bioinformatics computer-assisted search of publicly available databases, to identify and rank potential candidate genes according to a list of keywords. Of the 70 known or putative genes within the 5q 95% CI, 8 genes produced priority scores greater than 1 standard deviation (SD) from the mean priority score. Of the 107 genes within the 10q 95% CI, 13 genes produced priority scores greater than 1 SD from the mean priority score.

We are currently designing a detailed association screen of the 21 high priority genes in our two strongly implicated regions to identify genes underlying common migraine susceptibility. Such genes will provide clues to the further elucidation of the complex molecular pathways of migraine and, finally, will help in the development of diagnostic tests and rational treatment strategies.

Rachael Murray

Institute of Molecular Biosciences, University of Queensland.

SNAREing IMMUNITY

This work involves the study of immune cells, called macrophages, that defend the body against infection by 'eating' and killing disease-causing microbes. At the same time they release messengers that alter other immune cells. We have discovered a pathway within these cells that links these two actions and shows how the body responds so rapidly to an infection. When over-produced these messengers can cause chronic inflammatory diseases such as arthritis and psoriasis. This discovery provides new and exciting avenues for drug development to combat chronic inflammation.

SNAREing Immunity

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Macrophages are the sentinels of the immune system that provide both front line protection and disposal of invading pathogens by a process known as phagocytosis while simultaneously secreting a wide range of cytokines and chemokines that regulate and determine the nature of the whole body's immune response. TNFα is the earliest proinflammatory cytokine released by macrophages. It has an essential role in immunity but too much secretion of TNFα can be detrimental as seen in a number of chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease. Understanding the basic molecular and cellular machinery that drives both phagocytosis and cytokine secretion is important for our further understanding of immunity and will underlie new therapeutics for chronic inflammatory diseases. Both the upregulation of intracellular protein trafficking and movement of membrane to the cell surface are critical for both phagocytosis and secretion. When a macrophage encounters a microbe it engulfs it in membrane from internal sources, internalises, and degrades it, while at the same time synthesising and secreting TNFα. I have shown how these two processes are fundamentally linked. Within the cell newly synthesized TNFα, a transmembrane protein, is transported out of the endoplasmic reticulum (ER), to the Golgi complex, and out to the cell surface in a series of membrane-bound vesicular carriers for its secretion. Refuting the popular belief that membrane proteins traffic directly from the Golgi complex to the cell surface, I found that TNFα is transported in a two-step process via the recycling endosome. The efficient transport of TNFα to the cell surface requires the upregulation of key components of the trafficking machinery at strategic points en route, including specific members of the SNARE family of proteins that regulate membrane fusion, without which intracellular trafficking would not proceed. SNARE proteins mediate fusion at all membrane trafficking steps throughout the cell by forming protein complexes comprising of an R-SNARE on the vesicle membrane and 2 or 3 Q-SNAREs on the target membrane, with specificity being brought about by the location and partnering of these SNARE proteins. I identified a novel Q-SNARE complex on the trans-Golgi network and on the vesicles budding from this membrane containing TNFα (Murray et al, JBC 2005). These vesicles carrying TNFα fuse with the recycling endosome on their outward journey via this Q-SNARE complex and an R-SNARE, VAMP3, found on the recycling endosome. Membrane from the recycling endosome, containing TNFα is then sorted and trafficked to the cell surface, where the same VAMP3 mediates its fusion with a previously identified Q-SNARE complex located on the plasma membrane. Exactly how this pathway enhanced the rapid release of this early-response inflammatory mediator only became obvious when I began to study the phagocytosis of yeast. To overcome the loss of the cell surface membrane during the initial stages of phagocytosis internal membrane, including the recycling endosome, is inserted into the cell surface under the phagocytic cup. I found that TNFα is delivered to and secreted exclusively from the forming phagocytic cup via insertion of the recycling endosome (Murray et al, Science 2005). This clever hijacking of the recycling endosome allows the cell to rapidly release large amounts of TNFα from the cell while providing the excess membrane needed to engulf the microbe. Thus, the macrophage has uniquely adapted its trafficking mechanisms to ensure that the whole immune system responds to an infection in the most effective and rapid way possible. SNAREs at key strategic stages in this trafficking pathway are upregulated to increase the number of fusion events thereby efficiently delivering TNFα to the surface for secretion with the excess membrane needed to engulf a microbe. These molecules now provide novel targets for rational drug design to improve the treatment of chronic inflammatory disease.

Chris Schmidt

Queensland Institute of Medical Reseasrch

WHY DO SOME CANCERS SUCCUMB TO IMMUNE ATTACK?

Immunologists have long believed that cancers could be killed by the same kind of immune response that is so effective in overcoming virus infections. The evidence for this in humans has been poor. However, the successful use of anti-tumour "vaccines" in some patients with advanced metastatic cancers gives us the opportunity of learning more about this question. This kind of information is needed in order to improve the activity of these vaccines.

The most important actor in immune responses against cells (such as cancer cells) is the "T" cell. These can recognize very specific elements on target cells, called "antigens". One problem with immune responses to cancers seems to be that, if the cancer cell stops producing an antigen, T cells specific for the antigen won't see the cancer cell anymore.

We have developed an anti-cancer vaccine which is made from cells taken from the patient, and tested it in several clinical trials. We found that only patients with strong immune responses against their cancers also had lasting clinical responses. The cancer cells from these patients also had high levels of antigens that T cells could recognize, unlike the cancer cells from patients who responded poorly to vaccination. Therefore, to improve the responses of patients to vaccines, we may need to consider the biology of the target cancer cells, and their ability to evade immune attack.

The foe within. An immunological insight into the death of cancers

Chris Schmidt

Queensland Institute of Medical Research

Many patients believe that the key to curing advanced cancer lies within. Spontaneous cures are rare, but many studies show that the same complex system that has protected higher animals from viruses for hundreds of millions of years has the potential to eliminate tumours. And, unlike many toxic chemicals now in use, it can do so without leaving a trace of evidence, either of itself or of its foe. In these rare cases, the extraordinary fact is that usually, the cancer does not return. We remain ignorant of how immunity works, just as we misunderstand how it fails.

Cancers are organs that survive at the expense of other cells. If we can see them, they have clearly avoided the armies of T cells that apparently lie idle. But this hides the more subtle truth: we now speak of immunity "sculpting" the cancer, a true Darwinian struggle. The evidence for this is that cancer cells sometimes relinquish those surface features that make them recognizable to our sophisticated T cells. Even then, more primitive mechanisms of immunity must be overcome. Natural killer cells are capable of detecting changes in these recognition structures, but cancers can express decoys that mimic normality. The evidence of these changes in tumours suggests that the immune system may kill cancer cells at some stage, but the unequal battle has them lose sight of the enemy.

This raises the most important question: what allows the immune system to prevail? To answer this, we must find ways of coaxing the immune system into action. To avoid T cells, cancers can subvert the means by which they are activated. One way, of course, is simply to lie there, pretending to be normal – which, as far as the immune system is concerned, they pretty much are. Perhaps because of the local nuisance that the growing tumour causes, this can't last forever, but maybe long enough for the cancer to release its own toxins to inhibit those fragile, tentacled immune activists, the dendritic cells. This inhibition might be avoided if the dendritic cells were taken out of the patient's body (and so away from the cancer) long enough to nourish them with cytokines, arm them with cancer antigens, and poise them at the point of activating a waiting but ignorant army of T cells. We used this approach, loading patients' dendritic cells with their own cancer cells as the source of antigen. We reasoned that to distinguish friend from foe, those accumulated mutations peculiar to cancer might be the only effective flags for the T cells. And, we reasoned that repeated attacks would be necessary, as long as the cancer remained. In four separate clinical studies we have performed in collaboration with clinical investigators at the Mater and Royal Brisbane and Women's Hospital, a few patients have responded with complete regression of all detectable disease.

Luckily, we had immortalized some of the cancer cells to grow in our laboratory, so we could measure the immune response against them. You would think this was easy, but perversely most cancer cells do not grow where they are wanted. Melanomas, though, are different. We were surprised how strongly the patients' T cells reacted to their cancer, and how numerous they were. Even in the face of this immune onslaught, one patient took a year and a half finally to eliminate his cancer. In each case, this was achieved without major side effects. Only the patients who had complete tumour regressions had these strong T cell responses – patients who eliminated some of their tumours, only to have others grow elsewhere, had responses as low as those who showed no signs of regression.

They key to understanding why just these patients had such dramatic responses may lie in their cancers. Firstly, these patients had less disease; none survived that started with bulky cancers. And, it appears, their cancers did not play the evasive tricks mentioned above. Their cancer cells show no sign of losing the surface structures that allow them to be recognized by the immune system. So, of course, there is now another question: why would one patient's melanoma cells all remain intact, while another's become a faceless enemy? In science, there are no final answers. But, perhaps one day, finally a cure.