The Newsletter for the Society for Medicines Research

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New Secretariat for SMR

After many years with the Institute of Biology, we have a new secretariat. Just before Christmas last year, we were faced with the simultaneous resignations of both Christopher Ryan and Shirley Chan, who managed the administrative aspects of the SMR's activities. While we were assured this had nothing to do with the strains of organising SMR meetings, we were nevertheless left somewhat bereft while we sorted out alternative arrangements.

For the March 2000 meeting on Functional Genomics (see page 2), we owe a tremendous debt of gratitude to Malcolm Duckworth (SMR Secretary) and Geoff Stemp (SMR Treasurer), and to some additional secretarial help provided by SmithKline Beecham. Thanks to their substantial efforts, we kept the SMR going while we searched for an alternative provider of administrative services — capable and experienced in organising scientific meetings and running charitable societies.

We were very fortunate in finding Triangle 3 Ltd, which has taken over the Institute of Biology's role and added some of their own expertise drawn from managing other societies. Triangle 3 is wholly owned by the British Society for Immunology (BSI). Its profits are covenanted back to the Society each year and reinvested in the scientific and charitable objectives of the BSI. Triangle 3's secretariat team looks after a number of societies, including The Renal Association and The British Transplantation Society, and has experience in organising some very large meetings. Catriona Sanderson manages the small team of Helen Dempsey and Jane Roberts.

Jane will be your main point of contact and will maintain the membership database; please let her know of any changes to your details so that she can keep it up to date. Jane will also be assisting in organising the registration and venues for our meetings, so if you need information on these matters she should be able to provide it. You can contact the Secretariat by telephone on 0208 875 2431 or alternatively by e-mail on secretariat@socmr.org. We look forward to a long and effective partnership.•

Website developments

The SMR website continues to develop. It is now possible to pay by credit card for meeting registration, and we hope an encrypted transfer process will be permitted soon. For the moment, you can pay either using a non-encrypted transmission, or you can download a form and fax or post your registration through to the SMR secretariat.

Also on the website is a link to the webcasts of our meetings that are produced in collaboration with the specialist pharmaceutical publishers Prous. For some years we have published meeting reports in *Drug News and Perspectives* (indeed we will not abandon this paper form). The electronic version of the meeting includes an introduction to the field, a transcript of each presentation and a simultaneous audio version with slides. This latest venture was first pioneered with the 1999 Case Histories meeting; our second webcast is of the Apoptosis meeting held 13 July (see page 6). A link to each webcast is shown on the SMR home page (www.socmr.org).

We are very pleased with the result and believe it is a useful archive for those that attended, or for those that could not attend. But the presentations are obviously less imminent and more difficult to follow than a real physical presence allows. Besides, you miss all the networking possibilities — do let us know what you think!•

SMR COMMITTEE MEMBERS WANTED

Forthcoming committee member retirements in December 2000 will result in two vacant positions. Becoming part of the SMR committee presents an exciting opportunity for members to get involved with the running of the SMR and influence the meeting programme. If you are interested in applying, or have any questions, please contact the Secretary, Malcolm Duckworth on 01279 622017, or e-mail him on malcolm_duckworth@sbphrd.com. •

FUNCTIONAL GENOMICS

by Ray A. Jupp, Alan M. Palmer & Pam M. Greengrass

We are in the midst of a genomics revolution. The first chapter of this revolution will end later this year with the completion of the first draft of the entire human genome; estimates for the exact number of genes in the human genome vary between 50,000 and 140,000. This endeavour has been a major catalyst for the genomics revolution and has moved science into uncharted territories, requiring the establishment of both new disciplines and a new vocabulary. So, we now have pharmacogenomics, genotyping, pharmacogenetics, microarrays, biochips, differential display, bioinformatics and cheminformatics.

The SMR held a meeting on Functional Genomics at the National Heart and Lung Institute of the Imperial College of Science, Technology and Medicine on 1 March 2000. The meeting, attended by a multidisciplinary audience of various scientific backgrounds, provided both a review of some of the exciting progress that genomics has made of late, particularly in regard to its application to the discovery of new drugs, and a glimpse of what subsequent chapters of the genomics story may hold for the future.

Genomics

The application of microarrays (or DNA chips) to the drug-discovery process was reviewed by Dr Derk J. Bergsma (SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania, USA). Microarrays are small squares of thin glass on to which a set of probes are spotted capable of detecting the presence or absence of different genes. The microarrays are incubated with fluorescently labelled cDNA made from RNA isolated from cells or tissues. A confocal scanner is used to detect labelled cDNA hybridised to complementary DNA sequences on the chip. Fluorescent signal intensity represents relative expression of the corresponding genes when compared to their expression pattern in a control sample. Currently each microarray can detect between 7,000 and 10,000 different genes. It therefore provides a high-throughput technology to study RNA expression in cells and tissues (e.g. normal and diseased) of tens of thousands of genes simultaneously. As part of a team effort, scientists at SmithKline Beecham developed a robust cDNA-based microarray platform, and have validated the technology as a powerful tool for surveying RNA expression in human, yeast and bacterial systems.

Dr Bergsma described whole genome chips for *Streptomyces pneumoniae*, *Staphylococcus aureus*, *Saccharomyces cerevisiae* and HSV2 open-reading frames in addition to boutique grids for use in, for example, toxicology. SB have developed an automated tracking system for microarray data, querying and analysis. Several uses of the microarrays were provided. One use was to hunt for surrogate markers after treatment of cells with a therapeutic protein for tumour suppression. Another was to monitor *Streptomyces pneumoniae* genes affected by two component signal transduction knockout. This technology is still in its infancy and Dr Bergsma closed by identifying a need for better software tools and a need to develop techniques for faithful amplification of RNA when it is only available in very low levels.

Toxicogenomics

The identification of potential toxic liability in the drug discovery/development process is becoming increasingly important. A range of toxicity liabilities to be avoided, including rat carcinogenicity, rat teratogenicity, human chronic hepatotoxicity, human acute and chronic nephrotoxicity, human chronic neurotoxicity and human hypersensitivity. Dr Peter Bugelski (SmithKline Beecham Pharmaceuticals, Harlow) suggested that the purpose of toxicity screens is to provide an indication of possible toxicity rather than a definitive prediction of toxicity. He described the use of microarrays for toxicological assessment. This involves high-throughput genomic screening with an awareness that inherent in many screens are appreciable rates of both false positives and false negatives. Thus these screens should not become

'gatekeepers', but instead deal in probabilities.

He defined the characteristics of higher throughput screens to be >100 compounds per year; <100mg compound requirement; relatively inexpensive; reproducible over time; must provide a forecast of specified effect; and have defined rates of alpha and beta error. Two sorts of toxicological screens were described: SMART (Selective Markers Relevant to Toxicity, which are screens that test a specific hypothesis) and ESP (expressed sequence pattern, where a probability of toxicity is assigned by pattern matching) screens. Typically these screens measure expression of genes or gene products with defined function and are useful in selecting lead compounds with a low probability of a specific toxicity and are helpful in directing mechanistic studies. Using markers relevant to toxicity (for example P450 induction) it was argued that particular profiles of expressed sequence patterns could be used to provide a prediction of a toxicological liability in the clinic. The ESP screens are used to assign the probability of toxicity and pattern matching, comparing against a database containing mRNA fingerprints from appropriate cells or tissues. There is a need to refine microarray-based screens to identify genes associated with specific pathology. The lack of knowledge about gene expression in toxicity limits the SMART approach in many cases. The hope is that microarrays of appropriate genes will allow reproducible screening over time of greater than 100 compounds per year with a low compound requirement (<100mg) at relatively low cost (<$1,000 per compound total cost). It was argued that particular profiles of expressed sequence patterns could be used to provide a prediction of toxicological liability in the clinic.

Pharmacogenomics

Dr David Bailey (De Novo Pharmaceuticals, Cambridge, UK) defined pharmacogenomics as the science that combines pharmacology and genomics; this contrasts with the common meaning of this word, that is the tailoring drug treatment in the light of genomic variation, which he suggested could be better served by the name pharmacogenetics. Predicting that drug design would be the dominant technology of the 21st century, he went on to illustrate how conventional medicinal chemistry can be complemented by computer-aided drug design to provide both a virtual screen and *de novo* structure generation for both drug design and lead optimisation. The approach clearly works much better alongside 3D structural information. To illustrate this, it was shown how six families of GDP-binding proteins were used and structures compared on the basis of either similarity or dissimilarity. They have looked at 70 other therapeutic targets where there is crystal structure information. The emphasis is on the rapid speed which can be achieved compared with conventional methods. It was viewed as an appropriate tool for looking for second-generation compounds where, in the ideal case, the time taken going from a target structure to a drug should reduce from years to days.

Proteomics

The application of proteomics in the drug-discovery process was reviewed by Dr Martin Page (Oxford GlycoSciences, Oxford, UK). Genomics provides little information on proteins, their cellular localisation, post-translational modifications and protein-protein interactions. The relationship between mRNA and protein levels are unclear, and genomic/array analyses cannot easily study body fluids (such as serum, cerebro-spinal fluid, synovial fluid) where novel targets and markers may reside.

Genomic approaches have been complemented recently by the tremendous advances in the high-throughput systematic analysis of proteins, termed proteomics. This technique involves the separation of proteins using one- and two-dimensional polyacrylamide gel technology, detection of the proteins with highly sensitive fluorescent dyes, and then scanning the resulting gels to generate digitised images. Using landmarks, and advanced suites of software, the images are subsequently processed into a common geometry from which one is able accurately to interrogate the data, and identify those proteins differentially or uniquely expressed in different sample sets. Proteins of interest are subjected to trypsinolysis and tandem mass spectrometry, and the resulting spectra searched against databases in order to identify the proteins.

Much of this process is now automated, enabling the processing of hundreds of 2D gels and proteins per week. By the careful choice of sample types, and fractions thereof, it is possible to identify rapidly key disease-specific or regulated proteins, and to study biological processes; the output of which should have major impact across the entire drug discovery process.

The application of proteomics for new target and marker discovery was illustrated using examples from clinically derived breast-cancer tissue and sera studies. These studies are done as a collaboration between the Ludwig Institute for Cancer Research and the Cancer Research Campaign laboratories. Proteome analysis has been used to examine the differences at the protein level, between the two major cell types comprising the normal human breast. From the interrogation of over 43,000 proteins in this study, 170 proteins were identified whose expression differed significantly between the two purified cell populations. The derivation of an advanced composite proteome image of the breast luminal cells is particularly important, since the majority of breast tumours are known to arise from this cell type. In parallel studies, they have also derived proteome images from a series of purified cell preparations of primary and metastatic breast tumours. Comparisons of the proteomes from normal and cancerous breasts are under way, and disease-specific proteins have been found.

In addition to the cell-based studies above, proteome studies have been applied to fractionated material to generate an extensive database of human breast membrane proteins. Some of these proteins could be strong candidates for therapeutic intervention using antibodies or vaccines. These are areas where significant clinical data are emerging with encouraging results. The proteome studies have also been used to examine the proteins present in the sera from patients with primary breast cancer, metastatic breast cancer or normal human volunteers. Using a depletion process to remove the commonly abun-

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dant proteins in the sera samples, an initial pilot study involving sera from 17 patients in each of these groups was undertaken. Subsequent analysis of the differences between the groups identified clusters of proteins whose level of expression are altered. This pilot study has now been extended to encompass a longitudinal study, in which sera is examined from node-negative breast cancer patients who have either benefited from a period of extended remission, or who have relapsed early with recurrent advanced disease.

Bioinformatics

With the enormous amount of information now being generated from both genomic and proteomic studies, it is essential both to analyse and interpret these data. Dr Jean-Michel Claverie (CNRS-AVENTIS, Marseille, France) described use of non-sequence similarity-based bioinformatics approaches in functional genomics. There are three basic challenges for bioinformatics:

- finding the genes;
- locating their coding regions;
- predicting their functions.

However, our capacity for interpreting vertebrate genomic and transcript (cDNA) sequences using experimental or computational means lags far behind our raw sequencing power, even if the performance of current programs for identifying internal coding exons are good, the precise 5'→3' delineation of transcription units (and promoters) still requires additional experiments. Similarly, functional predictions made with reference to previously characterised homologues are leaving >50% of human genes unannotated or classified in uninformative categories ('kinase', 'ATP-binding', etc.). To overcome the insufficiency of sequence similarity-based bioinformatics, radically new approaches are being developed to take advantage of large-scale gene expression as well as comparative genomics studies, capable of providing genome-scale experimental information at a pace consistent with the progress of sequencing. Academic and industrial researchers are increasingly relying on those methods to prioritise their studies and choose their targets. For instance, the study of expression patterns can provide some insight into the function, reveal regulatory pathways, indicate side-effects of drugs or serve as diagnostic tools.

Jane Roberts, the new SMR contact at Triangle 3

Functional genomics

Extrapolating systemically from gene sequence to function is undoubtedly the major challenge facing industry and academia alike at the start of the millennium. Professor Igor Roninson (University of Illinois, Chicago, USA) reviewed a general approach for the identification and functional analysis of genes involved in almost any cellular process. The Genetic Suppressor Element (GSE) methodology permits identification of target genes through expression selection. GSEs are short gene fragments that show biological activity when expressed in a cell. In most cases, GSEs act either by encoding anti-sense RNA that inhibits the expression of the gene from which it is derived or by being translated as a dominant-negative peptide. GSEs can be used as specific genetic inhibitors of gene function, and as tools for identifying genes whose inhibition has the desired cellular effect. GSEs are generated by random fragmentation of target DNA, which may comprise a single cDNA clone, a viral genome, a subtracted cDNA population, normalised cellular cDNA, or genomic DNA. The mixture of target DNA fragments is cloned into a suitable expression vector, which is then introduced into the target cell type, followed by expression selection of biologically active clones.

GSE selection has been successfully used by different groups in bacterial, yeast and mammalian systems, using a variety of targets and selection strategies. The most efficient procedures for GSE selection in mammalian cells involve the use of retroviral expression vectors, which allow one to transduce up to 10^8 or more cells with a random fragment library, prior to selection of the desired phenotype. The integrated retroviruses can be efficiently recovered from the selected cells by PCR and tested for functional activity. Since the same cell can be infected with different retroviruses, combinations of functionally co-operating GSEs can also be identified.

GSE selection has benefited from the development of retroviral vectors for inducible gene expression. Such vectors allow one to select growth-inhibitory GSEs using, for example, the BrdU suicide selection technique. Other selection strategies that have been successfully used in mammalian cells are resistance to chemotherapeutic drugs, immortalisation of primary fibroblasts, neoplastic transformation of 3T3 cells and flow cytometric selection for altered antigen expression. Some published examples of GSE selection in mammalian cells include the development of efficient inhibitors that interfere with different biological functions of p53, isolation of GSEs that protect $CD4^+$ human cells from HIV infection, identification of kinesin as a determinant of drug sensitivity in human cells, and cloning of the ING1 gene that co-operates with p53 in its tumour-suppressor functions. The GSE approach continues to be used for the identification of potential therapeutic targets in cancer and other human diseases.

Dr D. Xu (University of Glasgow) provided an excellent illustration of functional genomics. Dr Xu described the identification and functional studies of differentially expressed genes between Th1 and Th2 cells. The Th1 and Th2 cell subsets have distinguished functions and play a central role in immunity. The development and balance of Th1 and Th2 cells can determine the outcome of many diseases. However, the mechanisms which control the differential function and balance of Th1 and Th2 cells in normal and patho-physological conditions are poorly understood. To identify selectively expressed genes which control the induction and function of Th1 and Th2

responses is a key immunological objective. There are implications in many diverse areas such as vaccination and immunotherapy of infectious and autoimmune diseases. By using differential display PCR, a collection of Th1 and Th2 specific genes has been identified.

One orphan receptor molecule, ST2L, a member of the IL-1R family, was stably expressed on murine Th2 but not Th1 cells. An antibody against ST2L strongly labelled Th2 but not Th1 cells. Three-colour single-cell flow cytometric analysis showed that cell surface ST2L was coexpressed with intracellular IL-4, but not with IFN-gamma. The antibody selectively lysed Th2 cells *in vitro* in a complement-dependent manner. *In vivo*, it enhanced Th1 responses by increasing IFN-gamma production and decreasing IL-4 and IL-5 synthesis. It induced resistance to Leishmania major infection in BALB/c mice and exacerbated collagen-induced arthritis in DBA/1 mice. Another member of the IL-1R family, IL-18R, was selectively expressed on Th1 but not on Th2 cells. An anti-IL-18R antibody inhibited IL-18- induced IFN-gamma production by Th1 clones *in vitro*. *In vivo*, anti-IL-18R antibody reduced local inflammation and lipopolysaccharide-induced mortality in mice. This was accompanied by shifting the balance from Th1 to Th2 responses, manifest as decreased IFN-gamma and proinflammatory cytokine production and increased IL-4 and IL-5 synthesis. Therefore, ST2L and IL-18R are the stable markers for Th2 and Th1 cells, respectively, and regulate Th1 and Th2 functions. These molecules may be targets for therapeutic intervention.

The use of model organisms for target validation in the pharmaceutical industry was considered by Dr Sohaila Rastan (SmithKline Beecham, Harlow). Why should a unicellular organism such as yeast be useful when studying human genes? What relevance has a gene found in the nematode worm to discovering new molecular targets for the pharmaceutical industry? Is a fly with a defective gene and an abnormal circadian rhythm *really* going to tell us anything useful about sleep patterns in man? The answer to all these questions is predicated on the premise that biological function is conserved during evolution as are the functionally significant regions of the genome. We can experiment with yeast, fruitflies, nematode worms and mice in a way that is not possible in humans and thus determine the molecular mechanism underlying the gene defect. Cross-species comparison and functional genomics in model organisms thus constitutes a powerful approach to identify genes and study their function in normal and pathological conditions.

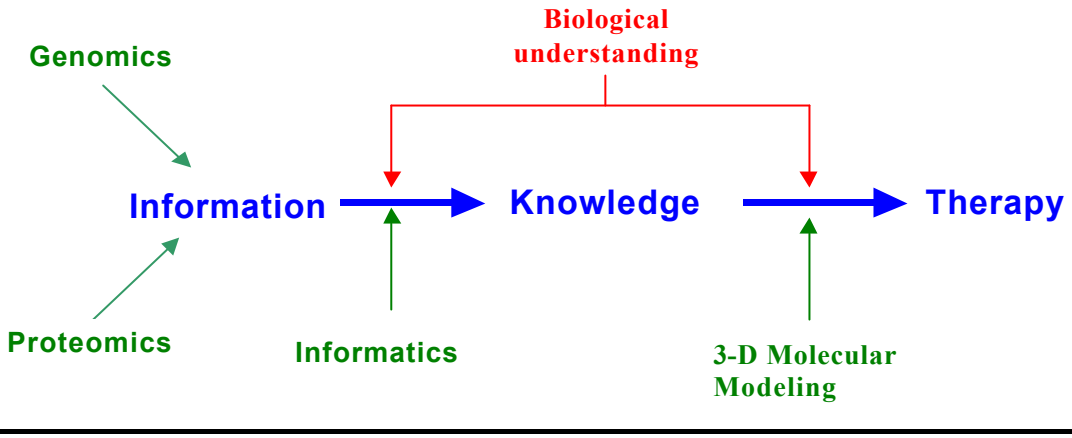

Figure 1: The central dogma of genomics and proteomics

Conclusions

Important hardware and software developments have generated a level of automation and throughput in both genomics and proteomics research that was inconceivable even ten years ago. The challenge facing us now is to *turn* the wealth of information now being accumulated into knowledge and then — possibly the greatest challenge — to *translate* this knowledge into new therapies (see Figure 1). This meeting succeeded in providing a glimpse of what the future holds. DNA chips (micro-arrays) are now being used for target identification, compound screening and the identification of surrogate markers. Similarly, the assessment of proteins is also being used to identify potential new targets and for the development of surrogate markers. It is clear that data at the level of both nucleic acid and protein are useful, but how well do they correspond?

Informatics are clearly essential to make sense of the wealth of information derived from both genomics and proteomics. It is highly likely that informatics will require further biological information, setting up an iterative process and a new type of hypothesis-driven research. Similarly, when translating the knowledge generated from this process into a new therapy (whether it is a small chemical entity, a gene or a large peptide) it is essential that an understanding of the associated biology is available. For these reasons, functional genomics is becoming increasingly important. The meeting reviewed a number of examples ranging from cells to whole organisms. The science of moving from gene to protein to drug is currently in its infancy, but it is clear that the use of 3D modelling of pharmacophores, particularly when linked with knowledge of the 3D structure of proteins, will be very useful in this exciting new approach to drug discovery. •

THE ROLE OF MITOCHONDRIA IN APOPTOSIS

by Alan M. Palmer, Pam M. Greengrass and David Cavalla

It has recently become apparent that mitochondria play a pivotal role in the process of cell death. In the absence of ATP, cells die by necrosis, but if sufficient ATP is available, a cascade of changes is initiated that leads to a much more orderly process of cell death (apoptosis). In addition to providing energy to the cell, mitochondria serve to sequester Ca^{2+}. Excessive accumulation of Ca^{2+} leads to the formation of reactive oxygen species, together with the opening of the mitochondrial permeability transition pore, which depolarises the mitochondria and leads to swelling. This may also provide a mechanism for the release of cytochrome c from the intermembrane space, although it is clear that there are probably other mechanisms as well. Cytochrome c normally functions as part of the respiratory chain, but when released into the cytosol it becomes a critical component of the apoptosis execution machinery, where it activates caspases (cysteine aspartate proteases) and (if ATP is available) causes apoptotic cell death.

The regulation of mitochondrial function by proteins related to bcl-2 was also discussed, together with the prospects for the development of new therapies for disorders associated with cell death.

Introduction

Cell death is a characteristic feature of neuro-degenerative disorders, such as stroke, traumatic brain and spinal-cord injury, Parkinson's disease, Alzheimer's disease and Huntington's disease, as well as ischaemic cardiac injury. There is, however, not yet any effective protective therapy. The need for such therapy is great and set to increase substantially in the years ahead since the incidence of many of these disorders (e.g. stroke, Alzheimer's disease, Parkinson's disease and coronary heart disease) increases exponentially after the age of 65, and the number of such people is set to double over the next 25 years. It is becoming increasingly clear that mitochondria play a central and critical role in the processes that lead to cell death.

The SMR's meeting on The Role of Mitochondria in Apoptosis, held on 13 July, brought together an international set of speakers, representing both academia and industry. It is now clear that mitochondria act not only as cellular powerhouses, but also play a decisive role in apoptotic and necrotic death pathways, including those relevant to acute and chronic neurodegeneration as well as cardiac ischaemia.

Three main themes of the meeting were:

- the pivotal role of mitochondria in cell death;
- the mechanism underlying apoptosis; and
- prospects for new therapies.

These are considered in more detail below.

The pivotal role of mitochondria

Excito-toxicity and mitochondrial impairment have been implicated in the pathogenesis of a number of neurodegenerative disorders. J. Timothy Greenamyre (Emory University, Atlanta, Georgia, USA) described how these two pathogenic processes are intertwined mechanistically. Mitochondrial dysfunction with ATP depletion impairs the activity of the Na^+/K^+-ATPase, which maintains neuronal membrane potential. As a result, the voltage-dependent blockade by Mg^{2+} of the N-methyl-D-aspartate (NMDA) class of glutamate receptor is reduced. In this setting, low concentrations of extracellular glutamate may become lethal. Additionally, mitochondrial impairment severely disrupts intracellular calcium homeostasis. Thus, mitochondrial dysfunction can produce secondary excito-toxicity. Mitochondria are also targets of excito-toxicity, such that NMDA receptor activation leads to mitochondrial swelling and the generation of reactive oxygen species.

Huntington's disease (HD) is a rare but devastating disease that provides one of the clearest examples of a chronic neuro-degenerative disease caused by mutations in a single gene. It occurs as the result of an expansion in the number of CAG repeats in the gene encoding the protein huntingtin. There appears to be a deficit in mitochondrial complex II in HD which may well cause secondary excito-toxicity. Thus, inhibitors of complex II (succinate dehydrogenase) such as malonate and 3-nitroproprionic acid, when administered into the striatum of rats, reproduce many aspects of the pathology of HD. Moreover, malonate when co-administered with NMDA caused much greater toxicity than either compound alone and this toxicity could be almost completely blocked by administering the NMDA receptor antagonist MK-801.

Activation of NMDA receptors caused a rapid increase in the concentration of cytosolic calcium, which had the same time course as the rise in mitochondrial calcium. This contrasts with the profile observed with kainic acid (where the rise in mitochondrial calcium occurred after the rise in cytosolic calcium) and suggests that mitochondria may well be closely associated with NMDA receptors in the neuron.

Mitochondria isolated from the fibroblasts of HD patients have a reduced capacity to accumulate calcium; with graded calcium loads, they depolarise more readily and recover less well than control mitochondria. Normal mitochondria can be induced to behave like HD mitochondria by incubating them with polyglutamine-containing proteins similar to the mutant huntingtin protein that causes HD. Exactly how an increased number of glutamines, recapitulates the bioenergetic defects and loss of calcium homeostasis remains to be established.

It has been estimated that Parkinson's disease (PD) affects over 1% of the population aged over 55. It is associated with progressive degeneration of dopamine neurons from the substantia nigra and has been linked to a selective defect in mitochondrial complex I. Indeed, systemic inhibition of complex I (with rotenone) produces a specific destruction of dopaminergic neurons in the substantia nigra rather than in the striatum. As the nigral dopamine neurons die, another group of neurons in the subthalamic nucleus becomes overactive. These subthalamic neurons are glutamatergic and project back to the dysfunctional dopamine neurons. It has been hypothesised that this increased glutamatergic drive upon already impaired neurons contributes to disease progression. Using a rat model

Preview of the Mitochondria webcast available at http://www.prous.com/mitochondria

of PD (injection of the toxin 6-hydroxydopamine into the striatum), Dr Greenamyre's group has found that chronic infusion of an N-methyl-D-aspartate (NMDA) antagonist (MK-801) and an AMPA/kainate receptor antagonist (NBQX) into the subthalamic nucleus reduces degeneration of nigral dopamine neurons by more than 50%.

The role of mitochondria in excito-toxic injury was further explored by Dr Ian J. Reynolds (Department of Pharmacology, University of Pittsburgh, Pennsylvania, USA) using various dyes to measure mitochondrial potential and oxidative stress. It is known that acute exposure of neurons to glutamate causes the activation of NMDA receptors and a massive influx of extracellular calcium through this receptor/ionophore complex. Much of this calcium is accumulated by mitochondria, and studies have shown that prevention of mitochondrial calcium accumulation protects neurons from injury, even though this manipulation results in very high cytoplasmic calcium concentrations. These data strongly suggest that mitochondria are the source of the signal that results in the death of neurons. The nature of this signal is not yet clear, but includes activation of caspase enzymes (see below) and mitochondrial generation of reactive oxygen species. The role of the mitochondrial permeability transition pore in this process is also not completely clear (see below).

In addition to illustrating what happens to mitochondria exposed to neurotoxic stimuli, Dr Reynolds' presentation also included an example of some unappreciated facets of normal mitochondrial function in the form of spontaneous mitochondrial depolarisations that can be observed in both neurons and astrocytes using dyes that report the mitochondrial membrane potential. This phenomenon was illustrated by a real-time video. As expected, the phenomenon was blocked by depletion of ATP stores and substantially enhanced by the addition of glutamate. The challenge ahead is to obtain quantitative information to explore this phenomenon further and permit assessment of the effect of drugs on the mitochondrial membrane potential.

The role of mitochondria in the initiation of both apoptotic and necrotic cell death was described by Dr Andrew Halestrap (Department of Biochemistry and the Bristol Heart Institute, University of Bristol). A major player in this process is the mitochondrial permeability transition pore (MPTP), a non-specific pore that opens in the inner mitochondrial membrane under conditions of elevated matrix (Ca^{2+0}), especially when this is accompanied by oxidative stress and depleted adenine nucleotides. These are exactly the conditions which occur during reperfusion of a tissue following a period of ischaemia and lead to 'reperfusion injury'.

Data were presented to indicate that an MPTP is formed through a calcium-mediated conformational change of the adenine nucleotide translocase (ANT), facilitated by bound cyclophilin-D (CyP-D). CyP-D binds tightly to the ANT following modification of critical thiol groups by oxidative stress which also cause adenine nucleotides to dissociate from their binding sites on the ANT. This model has recently been confirmed by the demonstration that the MPTP can be reconstituted into proteoliposomes using purified ANT and CyP-D. The extent of MPTP opening in the Langendorff perfused heart has been determined using mitochondrial entrapment of [^{3}H]-2-deoxyglucose. The MPTP does not open in the ischaemic heart, but does do so during subsequent reperfusion, although later in reperfusion it can close again and the extent of closure correlates with functional recovery of the heart. The implications of this for necrotic versus apoptotic cell death in reperfusion injury will be discussed.

A range of protocols that minimise opening of the MPTP were shown to protect both Langendorff and working hearts from reperfusion injury; these include addition of cyclosporin A, propofol (which has antioxidant properties) and pyruvate to the perfusion medium prior to ischaemia. Halestrap's studies have been with heart tissue, but collaborative studies with the labora-

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tory of Dr Tadeusz Weiloch suggest a similar role for the MPTP in the cell death occurring in the hippocampus following ischaemia/reperfusion or hypoglycaemia/ normoglycaemia. It was shown that in some systems (for example, the action of tumour necrosis factor a) cytochrome c release from mitochondria and induction of apoptosis may be independent of the MPTP. It is clear that there is much yet to be unravelled before we have a clear understanding of the precise role of the MPTP in cell death.

Mechanism underlying apoptosis

So mitochondria play a key role in the process of cell death and appear to release a signal (or signals) that initiate a cascade of changes that (if sufficient ATP is available) leads to apoptotic cell death. Understanding the nature of these 'death signals' was a theme taken up by Dr Seamus J. Martin (Department of Genetics, The Smurfit Institute, Trinity College, Dublin, Ireland). Apoptosis (programmed cell death) is co-ordinated by a family of cysteine proteases — the caspases — that dismantle the cell by cleaving a subset of cellular proteins after aspartic acid residues. Caspases participate in the molecular control of apoptosis in several guises: as triggers of the death machinery; as regulatory elements within it; and ultimately as a subset of the effector elements of the machinery itself. The mammalian caspase family is steadily growing and currently contains 14 members. Caspases were initially identified in *c.elegans* by comparing genes from cells that lived and those that died, a series of cell death genes were named Ced 3, Ced 4 which induced death and Ced 9 which prevented the action of Ced3/4. Other caspases include Bcl-2 (Ced 9 homologue), caspase 1 (Ced 3 homologue), APAL-1 and FLASH (Ced 4). At present, it is unclear whether all of these proteases participate in apoptosis.

It appears that numerous pro-apoptotic stimuli converge on mitochondria and provoke the release of factors (such as cytochrome c) that trigger caspase activation and cell death as a consequence. The main target of the initiating APAF-1/cytochrome c complex is caspase 9, which then activates caspases 3 and 7. Caspase 3 can then activate caspases 2 and 6, while caspase 6 can activate caspases 8 and 10. Evidence suggests a positive feedback loop whereby Bid is a substrate for caspase 3. Cleaved Bid is a pro-apoptotic protein which causes cytochrome c release and promotes the caspase activation process.

It is suggested that apical caspase activation events are initiated by molecules that promote caspase aggregation and facilitate auto-activation. Distal caspase activation events are controlled by caspases activated earlier in the cascade. Apical caspase activation is achieved via oligomerisation. Death receptors have adaptor proteins which can recruit caspases; the receptors trimerise and result in three caspases acting together. Apaf-1 and caspase 9 occupy non-redundant positions in the cell death pathway and Apaf-1 is selective for caspase 9. When cytochrome c is used in cell extract that has had caspase 9 removed, there is no triggering of other caspases. Caspase 9 is apical, while caspase 7 has no effect on any caspase activation event, caspase 3 removal still allows processing of caspase 2 and 6. Inhibition of caspase 8 and 10 has no effect on other caspases suggesting that caspase 3 is involved as a signalling caspase.

The concept of apoptosis was further elaborated by Dr John A. Hickman (Institut de Recherches Servier, Paris, France), who introduced other mechanisms that control the apoptogenic effects of mitochondria. Amongst these is the widely recognised Bcl-2 family of proteins, including the anti-apoptotic proteins Bcl-2 and Bcl-XL, and the pro-apoptotic proteins Bak, Bax and Bid, among many others. Regardless of the stimuli, a step-wise change in epitope availability occurs on the pro-apoptotic protein Bak in all cell types studied. Bak and Bax are pro-apoptotic whereas Bcl-2 and Bcl-xl inhibit apoptosis. All members of this family have a series of conserved domains, BH1, BH2, BH3 and BH4; Bcl-2 and Bcl-xl have a BH4 domain while Bax does not. The sequence of changes to these proteins initiated by damage is first the N-terminus where a cryptic epitope is exposed, possibly due to release of a protein that maintains Bak in a latent state. Bak is still bound to Bcl-xl and there is no evidence of apoptosis. A second change then occurs in which exposed central BH1 homology domain, Bak, is freed from binding to Bcl-xl, the N-terminus of Bax is exposed and the cells that activate caspases and apoptosis are evident. The BH3 domain of the anti-apoptotic Bcl-xl is exposed when the BH4 domain is cleaved by proteolysis making this pro-apoptotic.

Studies with a range of antibodies to specific regions in Bak have shown that all signals that result in cell damage cause N-terminus opening and that this initial step can be reversible. The Bcl-2 family members are pore-forming molecules by analogy with colicine. Helices lying between BH1 and BH2 domains form the 'pore'. Using a concentric double-barrelled patch clamp pipette, Dr Hickman described remarkable experiments that measured electrical recordings from mitochondria membranes in the intact squid giant axon pre-synaptic terminal. N-truncated Bcl-xl (resembling the BH4 cleaved protein) rapidly induced electrical activity at 200pA with a linear relationship between conductance and voltage while the full-length protein had very little activity, so it is clear that the BH3 domain is essential for channel activity. It was suggested that pro-apoptotic proteins are normally latent and require signals to expose the BH3 domain. These data suggest that it is the BH3 domain and not the pore-forming region with two a-helical domains, believed to lie between domains BH1 and BH2, which is responsible for channel activity. It is not clear if this very high-conductance channel will be big enough to permit the passage of a protein like cytochrome c, but regulation of the permeability of the outer membrane is likely to be important in controlling the release of cytochrome c.

The role of Bax in apoptosis was developed further by Dr Bruno Antonsson (Serono Pharmaceutical Research Institute, Plan-les Ouates, Geneva, Switzerland). He described how Bax associated with mitochondria from apoptotic cells is present as a high molecular weight complex whereas in mitochondria from non-apoptotic cells only Bax monomers can be detected. Bax is a Bcl-2 family protein with pro-apoptotic activity that can form ion channels in artificial lipid membranes. The localisation of Bax has been shown to change from the cytosol to the mitochondria during apoptosis. The protein can trigger cytochrome c release from mitochondria both *in vitro* and *in vivo*. There is evidence to show that the pro-

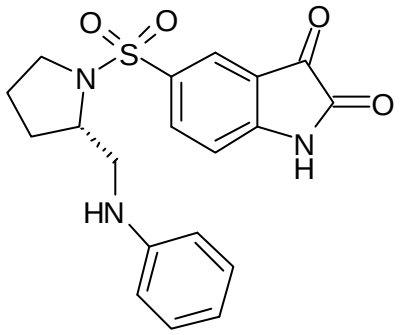
tein is present as a monomer not integrated in the mitochondrial membrane in untreated HeLa cells. After treatment with the apoptosis inducer staurosporine the monomeric cytosolic Bax concentration decreased and the Bax associated with the mitochondria is present as both a monomer and a large molecular weight complex, and is integrated into the mitochondrial membrane. The outer mitochondrial membrane protein VDAC did not co-migrate with the large molecular weight Bax complex. It was concluded that monomeric Bax is inactive in the mitochondria and Bax oligomerisation or complex formation with an unknown protein is required for activity.

Dr Antonsson also considered the prospects of using the Bcl-2 family of proteins as potential targets to develop new drugs. The Bcl-2 family contains pro- and anti-apoptotic members that provide strong leads for investigation of the apoptotic process. Bax structure illustrated conserved domains associated with apoptosis and transmembrane targeting. Three-dimensional structures of Bcl-xl and Bid were very similar with central helices and overall similarity to the diphtheria toxin. As this toxin was known to possess channel-forming proteins, it was reassuring to discover similar roles for the Bcl-2 family. Bax forms a multichannel with poor ion selectivity and no calcium dependency. Images of fluorescent-labelled Bax showed clear localisation in the mitochondria. Further studies with recombinant Bax showed it could be detected in oligomeric conformations after treatment with Triton X (but not other detergents). In mitochondria only oligomeric Bax releases cytochrome c and only in apoptotic cells was oligomeric Bax extracted. The trigger for oligmerisation was unknown but could involve association with other proteins including Bid. The model of apoptosis was then considered which included transition-pore formation and the rupture of the outer membrane and the release of cytochrome c. Large mitochondria and disrupted membranes were found after Bax treatment of cells. In contrast neuronal cell apoptosis was associated with small fragmented mitochondria which could be mimicked by Bax treatment and reversed by NGF. Bax, however, releases cytochrome c by several mechanisms and transition-pore formation should not be considered in isolation.

Prospects for new therapies

It is clear that mitochondrial defects and excito-toxicity play important roles in the pathophysiology of neuro-degenerative disorders such as HD and PD. In addition, apoptosis is also involved in infertility, renal and liver disease as well as AIDS. Dr Greenamyre reviewed therapeutic strategies targeting these processes that are likely to provide neuroprotection and slow disease progression, which include:

- alternative mitochondrial substrates;
- cofactors;
- creatine;
- blockade of the mitochondrial permeability transition pore;
- antioxidants;
- anti-excitotoxic agents;
- blockade of the apoptotic cascade.



SB-270913

The prospect of using mitochondria as potential drug targets is a theme that was developed by Dr Anne Murphy (Mitokor Corp, USA). She discussed the potential for therapeutic intervention in mitochondrial events associated with the control of cell death and chronic cell dysfunction. Mitokor employ a range of approaches and technologies including cellular cybrids, mitochondrial DNA sequencing, mitochondrial proteomics in silico cloning, and targeted basic research. The genomics effort is focused specifically on the human mitochondrial genome, which encodes for some three dozen mitochondrial proteins. There is a remarkable degree of variability in the mitochondrial genome between individuals in spite of its relatively small size, and investigators are currently assembling a database of human mitochondrial genome sequences to link phenotype to genotype in a range of diseases that may involve mitochondrial function.

The proteomics effort is similarly targeted to mitochondria, where the identity of a number of key proteins (such as the calcium uniporter, for example) remains unknown. These approaches were complemented by the use of cybrids to study mitochondrial function. Cybrids are prepared from mitochondria from donor platelets (no nucleus) and a Rho zero recipient (mitochondria DNA-free cell), these can be made to examine disease aetiology using patient mitochondria, examples were presented of both Alzheimer's disease and Parkinson's disease cybrids.

Dr Murphy also addressed the mechanism by which cytochrome c is released from mitochondria. Cytochrome c is located in the intermembrane space and has to traverse the outer membrane in order to bind to APAF-1 and activate caspases. Recent research suggests that some apoptotic signals induce the release of cytochrome c along with another intermembrane space protein, adenylate kinase, implying a non-specific release process. However, other apoptosis inducers result in cytochrome c release in the absence of translocation of adenylate kinase. These events occurred in the absence of evidence for mitochondrial uncoupling, suggesting that the permeability transition is not responsible for apoptogen release in this model. The data further imply that multiple mechanisms exist for the release of cytochrome c from mitochondria, opening additional potential avenues for targeted drug discovery.

Investigating potential mechanisms of apoptogen release focused on signals that induce the release of cytochrome c along with another intermembrane space protein adenylate kinase, to determine the specificity of the release process. It appears that the specificity of release depends on the nature of the apoptotic stimulus, so that staurosporin selectively releases cytochrome c, whereas thapsigargin and etoposide release both. Staurosporin appears to be the most effective activator of caspases, even though all three stimuli release cytochrome c effectively. Taken together, these data indicate that multiple mechanisms exist for the release of cytochrome c, and that there is much more to discover about this critical step in the initiation of apoptosis.

(continued on page 10)

(continued from page 9)

Another approach to the development of new therapeutics was described by Dr Michael F. James (SmithKline Beecham, Harlow), who considered the potential and problems associated with developing caspase inhibitors as neuro-protective agents. As caspases are latent and only activated during neuro-degeneration they would seem a good target and the predicted adverse-effects profile would be considered to be favourable. Caspases have been identified as homodimers or heterodimers that on cleavage re-mould and expose two catalytic sites. The catalytic clefts are formed from the small and large sub-unit and endow the enzyme with substrate specificity. Some elegant molecular models comparing the structure and charge distributions of the clefts in caspase 3 and 9 illustrated these interactions. Caspases were also differentiated biochemically with peptide inhibitors such as the tetra peptide ZVAD. Other inhibitors exist which are more selective for the caspase 1 and 3 families demonstrating up to 1,000-fold specificity.

In addition to these peptides natural caspase inhibitors exist such as poxins, serpins and baculvins. Dr James described the properties of SB-270913 — a potent inhibitor of caspase 3 that also inhibits caspase 9 at slightly higher concentrations. This compound is effective at low micromolar concentrations against several forms of neuronal injury, including oxidative injury of HT4 cells, and neurotoxicity models involving primary neuronal cultures from the rat, but interestingly has no activity in an NGF-withdrawal model of neural injury — the desirable properties of ideal protease inhibitors. Dr James highlighted some of the pitfalls associated with targeting caspase enzymes. Not the least of these is the fact that the compounds must work intracellularly, which means that the dose has to be higher and so the likelihood of side-effects increases markedly. Another complication arises from the multiplicity of the interactions, for example, caspase 3 activates the other caspases while the role of other pathways are just starting to emerge.

Dr James then went on the describe a model of neuro-degeneration using cerebral arterial occlusion to invoke ischaemia which involves a wide range of pathological processes such as apoptosis and inflammation. In this model he showed that in caspases 3, 7, 8 and 11, mRNA is expressed during lesion formation and formatted a mechanistic basis for the alleviation by caspase inhibitors. These results were mirrored in hippocampal slices that have an advantage in that the neuronal electrical activity can be monitored at the same time as the biochemical changes. Higher screening throughput was obtained using a variety of neuronal cell lines that demonstrated promising site specificity even though the potency of the inhibitors was not impressive.

The early stage of development and great promise of this field was reflected in the lively discussion of the talk. One question of particular relevance was the systemic reaction to the inhibitors by tissues in which caspases were constitutively active. Will tumours arise? Clearly these considerations must borne in mind in the development of these compounds.

Conclusions

Under normal circumstances, mitochondria serve an essential bioenergetic role within the cell and (it is now recognised) that they also serve to regulate the concentration of free cytosolic calcium. Following cell injury (as a result of trauma, ischaemia or other disease mechanism), and in contrast to its primary life-giving role, mitochondria play a central role in the processes that lead to cell death. Whether a cell dies quietly (by apoptosis), or in a fashion that lets all its neighbours know about it (by necrosis), depends on the amount of ATP available. It is clear that mitochondria send one or more signals (e.g., cytochrome c) to the cytosol to initiate the apoptosis execution machinery, where it activates the cascade of caspase enzymes. The meeting provided a useful overview of this rapidly expanding field of research and introduced some of the approaches being used to develop new drugs targeting mitochondria or caspase inhibition. Given that mitochondrial dysfunction is likely to be a universal mechanism in cell death, it does seem likely that understanding the role of mitochondria in apoptosis is likely to provide a rich source for future drug discovery aimed at preventing cell death. •

SMRx Notes

DIARY

4–8 September 2000. International Symposium on the Chemistry of Natural Products, Sao Paulo, Brazil. Contact: Dr M. Fatima or G. F. da Silva, Universidade Federale de Sao Carlos, Depto de Quimica, Via Washington Luiz, km 235, Sao Carlos, CP676 Sao Paulo, Brazil. Tel: +55 16 274 8208; fax: +55 16 274 8350; e-mail: dmfs@power.ufscar.br.

2–5 October 2000. The Society for Biomolecular Screening 6th Annual Conference and Exhibition, Monterey, CA, USA. Contact: The Society for Biomolecular Screening, 36 Tamarack Avenue, Suite 348, Danbury CT 06811, USA. Tel: +1 203 743 1336; fax: +1 203 748 7557; e-mail: website@sbsonline.com.

12–13 October 2000. Pharmacokinetics and Pharmacogenomics, Institut Pasteur, Paris, France. Contact Institut Pasteur, 25–28 Rue du Docteur Roux, 75724 Paris, Cedex 15, France. Tel: +33 1 45 68 80 91; fax: +33 1 40 61 30 25.

15–18 October 2000. ISLAR 2000 — The 18th International Symposium on Laboratory Automation and Robotics, Boston, MA, USA. Contact Christine O'Neil, ISLAR 2000, 68 Elm Street, Hopkington MA 01748, USA. Tel: +1 508 497 2224; fax: +1 508 435 3439; e-mail: islar@islar.com; website: http://www.islar.com.

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