Session 7.3
The contribution of the OMICS technology to the 3Rs

Lecture

Characterisation of *in vitro* cultures of primary cells by expression profiling

Hans Jürgen Ahr, Heidrun Ellinger-Ziegelbauer, Grazyna Wasinska-Kempka and Christina Weiland
Bayer HealthCare AG, Molecular and Special Toxicology, Wuppertal, Germany

The toxicological evaluation of drugs and chemicals still relies almost exclusively on studies in animals. However, both ethical and economic reasons strongly advocate to find alternative animal free methods, which may at least in part replace these *in vivo* studies. *In vitro* cell culture systems of different organs and species including humans are widely considered as the method of choice in this respect. It is however, necessary to clearly understand the advantages, but also the limitations of such *in vitro* systems before they can be used for specific purposes. The recently developed gene expression techniques using microarrays now offer an unprecedented opportunity for the characterisation of these cell systems. This technology allows the simultaneous analysis of mRNA expression levels of thousands of genes, thus enabling a broad insight in the functional state of cells in culture as compared to their *in vivo* counterpart. Examples will be given of the expression patterns of cells of different origin (liver, kidney) in culture in comparison to the *in vivo* situation. The functional limitations of cell culture systems will be exemplified under the influence of model toxins. The findings will then be discussed with respect to the reliability of *in vitro* systems for toxicological testing of compounds.

Lecture

Detection of pain and stress by monitoring gene expression

Paolo Cinelli, Margarete Arras and Kurt Bürki
University of Zurich, Institute of Laboratory Animal Science, Zurich, Switzerland

One of the main concerns with regard to animal experimentation is that, if animals must be used for experimental purposes, pain and distress should be abolished or reduced to an absolute minimum. Considerable progress has been made concerning the application of the principles of reduction and replacement, however to implement refinement it is necessary to improve our ability to objectively recognise signs of pain and distress.

Pain can be thought of as having both sensory (discriminative) and affective (the unpleasantness) dimensions, and is usually classified as acute or chronic depending on its duration and as neuropathic when it derives from direct damage to the nervous system. Molecular dissection has begun to reveal distinct functions for these separate pathways and their contribution to the final behavioural outcome. Specific patterns of phenotypic change characterise different chronic pain conditions and it is these distinct molecular signatures that need to be considered if effective pain detection and control have to be achieved.

DNA microarrays are among the most powerful and versatile tools for genomics and genetics research. The main goal of our project is the identification of a set of genes that change their expression levels during pain and stress conditions in the mouse. With a pool of selected genes we designed a low density microarray and monitored changes in gene expression levels in mice under different pain/distress conditions. Our results indicate that the genes present on the microarray are useful for an objective detection of different pain levels in the mouse.

Lecture

Gene expression as the basis for alternatives methods: Estrogens as an example

George Daston, Jorge Naciff, Gary Overmann, Leslie Foertsch and Zubin Khambatta
Procter and Gamble, Miami Valley Labs, Cincinnati, USA

Microarrays have made it possible to evaluate gene expression on a scale that had not been technically feasible before. Changes in gene expression are sensitive indicators of biological response and specific patterns of changes can identify toxicological mechanism. Evaluation of gene expression can be added to traditional toxicity tests as a refinement, to gain more and better information, can reduce the number of animals used in testing, or as the foundation for *in vitro* replacement. Each of these is illustrated using endocrine disrupter (ED) screening as an example. Estrogens of varying potency produce a characteristic profile of gene expression in reproductive tissues of rat fetuses. The dosing protocol used is comparable to the OECD 414 assay. Evaluation of gene expression can be added as a refinement that makes the assay a screen for ED as well as a developmental toxicity. It is also possible to evaluate gene expression in a limited number of animals as a substitute for the mammalian portion of the USEPA's ED screening battery, resulting in a marked reduction in animals. Finally, gene expression provides a more specific readout of hormonal activity in cell-based assays. We have evaluated gene expression in MCF-7 cells (breast cancer-derived) after estrogen exposure, and are comparing these results to human uterine cells. The causal relationship between gene expression and higher order effects, along with its conservation across species, makes it an excellent basis for a 3R approach to alternatives development.

Poster

"Omics" technologies enabling the 3Rs in drug discovery and development: Treating human disease by studying humans

Sadhana Dhruvakumar
People for the Ethical Treatment of Animals (PETA), Research and Investigations, Norfolk, VA, USA

The first step of drug discovery is "target identification" in which the disease state is studied to identify a molecular target for modulation through chemical intervention (i.e., a drug). Until recently, target identification relied heavily on finding targets by studying animal models of human disease (natural or engineered). However, animal-based targets are only successful to the extent that the relevant biology is replicated in humans – a hit-or-miss proposition due to differences between species and between the animal and human diseases. However, a new paradigm is emerging as genomics, proteomics, and other "omics" technologies are facilitating the molecular level study of humans. Targets are increasingly found by studying human tissue (e.g., normal *vs*. diseased or early *vs*. late stage) for differential gene or protein expression to identify genes that are involved in the disease process. Cellular pathways associated with the human disease can be mapped using such techniques, leading to the identification of high-quality human-relevant disease-specific targets. Another application of "omics" technologies comes later during drug development, when these technologies can be used to identify early biomarkers of drug efficacy or toxicity. Early stage human drug testing or "experimental medicine" utilising such biomarkers is increasingly popular and is replacing some animal preclinical testing. The identification of "omics" biomarkers is also being done within animal experimentation. While this enables more humane endpoints, the fact remains that attempting to extrapolate from animal-based human drug research is inefficient compared to studying humans directly, and resources should shift more quickly towards human-based "omics" approaches.

Poster

The use of toxicogenomics in risk assessment: Perspectives and challenges

Ursula Gundert-Remy, Hans-Bernhard Richter-Reichhelm and Axel Oberemm
Federal Institute for Risk Assessment, Safety of Substances and Mixtures, Berlin, Germany

Technologies have been established that generate complex information on molecular changes in animal and man following exposure to chemicals. Having a greater understanding of this information alongside, empirical toxicological reference data provides for the continued evolution in our ability to understand toxicological mechanisms of action, thereby providing a more scientific basis for species extrapolation. Performing toxicogenomic analyses in traditional short-term animal studies will improve the quality of information available from such studies, leading to reduction of possible follow-up studies. In addition, toxicogenomics will aid the identification, development and validation of improved *in vitro* alternatives. On the other hand, toxicogenomics also provides specific opportunities for improvements at different stages of the risk assessment process such as the development of new predictive models for identifying human health hazards and more significant molecular biomarkers of exposure. Molecular profiling can be used for screening of existing chemicals and to quickly identify potentially hazardous substances and to categorise chemicals and mixtures of chemicals into different mode of action groups. As there is indication that molecular signals differ at dose levels, it is hoped that toxicogenomic information can also contribute to the understanding and interpretation of effects seen with low dose exposure. Gene polymorphisms are known to play a role in the different intra-species susceptibilities to chemicals, thus explaining for the observed differences in effects. Growing knowledge of genomic variability will enable a greater insight into the factors behind the observed variability in susceptibility to chemical exposure that can be seen in human populations.

Lecture

In vitro models and quantitative differential proteomics technologies for molecular signatures of neurotoxicity and neuroprotection

*Martina Klemm and André Schrattenholz**
ProteoSys AG, Mainz, Germany

New challenges for hazard and risk assessment in pharmaceutical and chemical industries and problematic animal testing require the development of a human *in vitro* models for the molecular characterisation of drug effects. The outstanding potential of human Embryonic Stem Cell (hESC)-based *in vitro* models is put in context with results from corresponding murine ESC-screening system. In combination with quantitative differential proteomic display techniques, biomarkers for neurotoxicity are developed. Results are superior to those of conventional array technologies (nucleic acids), because the proteomic analysis covers posttranslational modifications.

The main task of a comprehensive analysis of proteins ("proteomics") is the establishment of a reliable methodology for complexity reduction. Here we present data from experiments with embryonic stem cells during conditions of neuronal stress and rescue, demonstrating the feasibility of quantitative pattern control of complex samples.

Taken together proteomic strategies presented here are able to reliably and quickly detect and identify key molecular events in mode of action or toxicological studies, providing information which is not accessible by standard array technologies, which currently only display the amino acid backbone information of proteins (cDNA or recombinant protein) without detecting post-translational modifications and thus missing important functional details. Given the enormously complex and dynamic nature of these modifications, and the redundant and pleiotropic organisation of almost all major signal transduction pathways, we envisage the emerging importance of protein signatures of functionally related sets of posttranslational protein isoforms, rather than single targets or surrogate biomarkers.

Poster

Toxicoproteomics – Identification and pre-validation of potential early biomarkers in chemically induced hepatocarcinogenesis

Michaela Kroeger[1], Kerstin Fella[1], Matthias Glueckmann[2], Volker Kruft[2], Juergen Hellman[1] and Peter-Juergen Kramer[1]

[1] Merck KGaA, Institute of Toxicoloy, Darmstadt, Germany; [2] Applied Biosystems, Support Proteomics, Darmstadt, Germany

A common animal model of chemical hepatocarcinogenesis was used to identify early protein biomarkers as an alternative to the classical toxicological endpoints. N-nitrosomorpholine was administered to male Wistar rats for 7 weeks followed by an exposure-free period of up to 25 weeks. Five animals per group were sacrificed at different time points during and after exposure.

Proteome analysis is mainly based on the separation of proteins by 2D-gelelectrophoresis (2DE) as a first step prior to characterisation by mass spectrometry (MS). By using 2DE, many differentially expressed proteins could be detected after 3 weeks as well as 25 weeks of NNM treatment in rat liver tissue. Subsequent MS analysis has been able to identify most of these proteins. The intersection of differentially expressed molecules in both time-points revealed that many endpoint related proteins of week 25 are already detectable in week 3. For verification of these potential early biomarkers identified by 2DE/MS, we have utilised an alternative MS-based protein quantitation method, the iTRAQTM reagent technology. Many of the proteins deregulated in week 3 could be confirmed by this method. These results show that detection of early protein biomarkers is possible with proteomic approaches. Whether these new biomarkers can support predictive toxicology in order to improve and shorten regulatory carcinogenicity studies is under further investigation.

Lecture

Gene expression analysis of responses to xenobiotics of primary rat hepatocytes in double-layered co-culture systems with small intestinal cells

Masaki Nishikawa[1], Nobuhiko Kojima[1], Emiko Kitagawa[2], Hitoshi Iwahashi[2], Takatoki Yamamoto[1], Teruo Fujii[1] and Yasuyuki Sakai[3]

[1] Institute of Industrial Science, University of Tokyo, Tokyo, Japan; [2] National Institute of Advanced Industrial Science and Technology, Human Stress Signal Research Center, Tsukuba, Japan; [3] Center for Disease Biology and Integrative Medicine, University of Tokyto, Tokyo, Japan

Introduction: Usual cytotoxicity tests do not include important metabolic processes such as absorption or biotransformation. To overcome this problem, we proposed physiologically-based simple double-layered co-culture system using cell lines representing the small intestine and liver tissue, where we observed enhanced metabolic functions of the liver by the possible interactions between the two cell lines. In this study, to verify physiological relevance and the mechanisms responsible for this metabolic enhancement, we investigated DNA microarray analysis of primary rat hepatocytes co-cultured with rat small intestinal cells, IEC-6.

Methods: We co-cultured IEC-6 and primary adult rat hepatocytes using a highly-O_2-permeable material, polydimethylsiloxane (PDMS), for the hepatocytes culture. Next, we analysed the poly-cyclic aromatic hydrocarbons (PAHs)-triggered alterations of gene expressions in the hepatocytes using DNA microarray and compared them with those of *in vivo* oral administration.

Results and discussion: The improved double-layered co-culture system using PDMS successfully maintained the functions of hepatocytes beneath the IEC-6 membranes, whereas conventional double-layered co-cultivation failed to sustain the hepatocytes viability by the lack of O_2 supply. DNA microarray analysis demonstrated IEC-6 has many different influences on hepatocytes activities, such as cell attachment, fat or fatty acid metabolism, or hormone responses. In addition, we observed better *in vivo*-mimicking gene expression profiles in co-cultured hepatocytes indirectly attacked by PAHs through the IEC-6 cell layers than those in pure culture hepatocytes directly attacked by PAHs without IEC-6 cells. These results show the potential of the co-culture system in *in vitro* estimation of *in vivo* human responses when xenobiotics are orally administered.

Poster

Identification of potential markers for an *in vitro* test system of renal carcinogenesis

Kerstin Stemmer[1], Judith Hähnlein[1], Heidrun Ellinger[2], Hans J. Ahr[2] and Daniel R. Dietrich[1]*

[1] University of Konstanz, Environmental Toxicology, Konstanz, Germany;
[2] Bayer Healthcare AG, Molecular and Special Toxicology, Wuppertal, Germany

The explanation of the toxicological potential of a substance currently requires large numbers of animals. One promising approach for an *in vitro* alternative is the application of microarrays to elucidate sets of early toxic insult markers with high correlation to *in vivo* systems. The aim of our study is the development of an *in vitro* pre-screening system, using primary and continuous renal cell cultures to characterise the carcinogenic potential of substances in the kidney. In a first phase of this study, male Eker rats were orally treated with the renal carcinogen aristolochic acid (AA). After 1, 3, 7, and 14 days of exposure, three animals from each group were sacrificed, RNA from the kidney cortex was isolated and gene expression profiles were analysed on Affymetrix RAE230A chips. Characteristically deregulated genes were extracted and functionally annotated using statistical and clustering tools. DNA-damage repair genes including p21 and MGMT were up-regulated in all exposure groups. Real-time PCR, allowed the verification of AA-dependent deregulation of both markers in NRK-52E and primary kidney cells from F344 rats. Since DNA damage and deficiencies in the corresponding repair are integral in the process of cancerogenesis, both genes represent promising *in vitro* markers and should be validated in further cell culture systems from different species.

Poster

A novel TOSHI scaffold useful for inducing cell behaviour and its application to the cellomics study

Toshiaki Takezawa and Tomoyo Takeuchi
National Institute of Agrobiological Sciences, Laboratory of Animal Cell Biology, Tsukuba, Japan

We established a novel concept for the cellomics study to systematically analyse cell behaviour and phenotypes by culturing various cells on the TOSHI (tissue/organ section for histopathology) scaffold reflecting tissue conditions *in vivo*.

We noticed that thin tissue sections commonly prepared for histopathology retained the original microarchitecture and composition of the tissue, developed a breakthrough technology of culturing animal cells on a TOSHI scaffold, and demonstrated the application of TOSHI scaffolds made of a bovine placenta in tissue reconstruction and a serum-free culture. The labyrinth region of the scaffold induced unique cell behaviours to form multicellular spheroids of BeWo cells (human choriocarcinoma cell line), a capillary network-like structure for CPAE cells (bovine pulmonary artery endothelia), and a neuronal network-like structure for PC-12 cells (rat pheochromocytoma cell line). The scaffold provided a microenvironment to maintain the viability of PC-12 cells in a serum-free condition. Also, we succeeded in preparing a multicellular mass of NHDFs (normal human dermal fibroblasts) involving acellularised section-derived components.

TOSHI scaffolds conserve many of the biochemical factors that serve as signalling cues for inducing cell behaviour and phenotypes, and those factors are easily detected by ordinary techniques such as immuno-histochemistry or *in situ* hybridisation. Also, TOSHI scaffolds can be prepared not only from all animal tissues/organs of any age regardless of pathology, but also from the entire bodies of small animals. Taking these advantages, the analysis of interactions between different cell types and various TOSHI scaffolds will play an important role for a novel approach to study cellomics.

Poster

3R potentials of "-omics" technologies

Marjolein van Boxel, Marian Moelands and Coenraad Hendriksen
Netherlands Centre Alternatives to Animal Use, Faculty of Veterinary Sciences, Utrecht University, Utrecht, the Netherlands

In a relatively short time-span, biomolecular engineering has evolved into a scientific discipline with substantial promise to revolutionise biomedical research. High-throughput genomics, proteomics and metabolomics technologies enable simultaneous monitoring of the expression of large numbers of individual genes and proteins, resulting in a more profound mechanistic insight in (patho-) physiological processes. Great advances can be made in a wide variety of research areas, such as for drug discovery, vaccine development and safety assessment of chemicals. Little was known about the consequences of these new methodologies for laboratory animal use, although some technologies will probably initiate scientific questions that require (additional) animal experimentation. Apparently, though, "-omics" methodologies may also have potentials for the replacement and, notably, the reduction and refinement of animal experimentation. Increased predictability of tests enables the application of earlier, more humane endpoints. Animal testing may be shifted to later stages in the drug development process and low, subclinical doses of chemicals/drugs can be studied. More and scientifically more relevant data may be attained from a smaller number of animals, due to a better general set-up and a science-based selection of the most appropriate animal model. In co-operation with the Netherlands Genomics Initiative (NGI), the Netherlands Centre Alternatives to animal use organised the first international conference on the potentials of genomics technology as a 3Rs tool, "Genomics & Alternatives to animal use 2004". A presentation will be given of the most important results of this conference, which was held in June 2004 in Maastricht.