Session 5.7
Progress in quality assurance for *in vitro* alternative studies

Poster

A validated novel method to quantify angiogenesis *in vitro*

Mahtab Bahramsoltani and Johanna Plendl
Institute of Veterinary Anatomy, Freie Universität Berlin, Berlin, Germany

Angiogenesis, defined as sprouting of new capillaries from pre-existing ones, occurs in a cascade of migration, proliferation, differentiation and three-dimensional organisation of endothelial cells. Angiogenesis is a pre-requisite for growth and differentiation of organs and tissues and is involved in many pathological processes, for example growth and metastasis of tumours.

Pro- and anti-angiogenic factors are tested in numerous *in vivo* and *in vitro* models of angiogenesis. However, in these models, effects of the substances tested were quantified in only a few phases of angiogenesis.

The aim of this study was to establish and validate a method to quantify all stages of angiogenesis and anti-angiogenesis *in vitro*. Endothelial cells isolated from slaughtered cattle were incubated in specific medium. Angiogenesis up to the formation of lumenised capillary-like structures was examined by phase contrast and electron microscopy. Both morphological and ultrastructural changes of cells showed analogies to angiogenesis *in vivo*. By precise staging of the cellular alterations the entire angiogenic cascade was quantified. The reproducibility of quantitation of angiogenesis was verified by examination by different persons and in different culture dishes. Statistical evaluation showed that reproducible quantitation was possible by different persons and in a small sample size.

In conclusion, the present *in vitro* model allows a viable quantitation of angiogenesis and anti-angiogenesis *in vitro*. It can be employed either in trial studies of potential angiogenic and anti-angiogenic substances, respectively, or in the investigation of their cellular mechanisms and may thus provide an efficient method to reduce animal testing.

Lecture

The Importance of Good Cell Culture Practice (GCCP)

Michael Balls [1], Sandra Coecke [1], Gerard Bowe [1], John Davis [1], Gerhard Gstraunthaler [1], Thomas Hartung [1], Robert Hay [1], Otto-Wilhelm Merten [2], Anna Price [1], Leonard Schechtman [1], Glyn Stacey [1] and William Stokes [1]

[1] European Centre for the Validation of Alternative Methods (ECVAM), Institute for Health and Consumer Protection, European Commission Joint Research Centre, Ispra, Italy; [2] Consumer Protection, European Commission Joint Research Centre, Ispra, Italy

The maintenance of high standards is fundamental to all good scientific practice, and is essential for maximising the reproducibility, reliability, credibility, acceptance and proper application of any results produced. Following the publication of outline guidelines for Good Cell Culture Practice (GCCP) after the 3rd World Congress on Alternatives and Animal Use in the Life Sciences (Bologna, Italy, 1999), a new task force was convened by ECVAM, with a broader range of expertise in cell and tissue culture, in order to produce an updated and more-detailed GCCP guidance document for practical use in the laboratory.

This GCCP Guidance, which will have been published in *ATLA* and made available elsewhere before the Berlin Congress, is based on the following six operational principles:

1. Establishment and maintenance of a sufficient understanding of the *in vitro* system and of the relevant factors which could affect it.
2. Assurance of the quality of all materials and methods, and of their use and application, in order to maintain the integrity, validity, and reproducibility of any work conducted.
3. Documentation of the information necessary to track the materials and methods used, to permit the repetition of the work, and to enable the target audience to understand and evaluate the work.
4. Establishment and maintenance of adequate measures to protect individuals and the environment from any potential hazards.
5. Compliance with relevant laws and regulations, and with ethical principles.
6. Provision of relevant and adequate education and training for all personnel, to promote high quality work and safety.

Lecture

Macroscopic evaluation of HET-CAM biomaterial testing: How reliable is macroscopical scoring without histology?

Claudia Eder [1], Erwin Falkner [2], Helmut Appl [3], Harald Schöffl [3], Udo M Losert [2] and Stefan Nehrer [1]

[1] Medical University Vienna, Dept. of Orthopaedic Surgery, Vienna, Austria; [2] Medical University Vienna, Core Unit for Biomedical Research, Vienna, Austria; [3] ZET-Centre for Alternative and Complementary Methods to Animal Testing, Linz, Austria

The high vascularity and the rapid development of connective tissue and vessel system of the chick chorionallantoic membrane offer an interesting environment for tissue reaction studies, which are often evaluated by macroscopical examination only.

Various biodegradable scaffolds were applied onto the CAM and maintained *in ovo* for 3 days prior to digital documentation, macroscopical biocompatibility evaluation and subsequent histological analysis. A collagen sponge, two different Collagen Type I/III scaffolds (Chondro-Gide®, Bio-Gide®) and a Collagen Type II membrane (Chondrocell®) were tested.

Collagen sponge: Macroscopic analysis demonstrated extreme rapid degradation, spontaneous bleedings in the surrounding of the implant and a vessel retraction from the implantation site. Histological analysis, in contrast, demonstrated an increase in blood vessel content. A foreign body tissue reaction was observed only histologically.

Chondro-Gide®: Macroscopic evaluation showed excellent integration and biocompatibility patterns which were confirmed by histology. Bio-Gide®: Macroscopic observation showed excellent integration and significant induction of angiogenesis, which was confirmed by histology. An inflammatory infiltrate was observed in histological sections only. Chondrocell®: Spontaneous bleedings at the implantation site as well as vessel retraction and altered vessel courses were observed macroscopically. Histological evaluation in contrast demonstrated good angiogenetic properties.

Macroscopical scoring only partially correlated to histological evaluation: The impact of spontaneous bleedings and vessel path alteration was often overestimated and a foreign body tissue reaction could not be detected after macroscopical evaluation. HET-CAM biomaterial testing should therefore be combined with histological evaluation to receive the full force of expression of the CAM model.

Lecture

Varioscope-head mounted microscopy for HET-CAM applications

Erwin Falkner[1], Claudia Eder[2], Helmut Appl[3], Udo Losert[4] and Harald Schoeffl[5]

[1] Core Unit for Biomedical Research, Medical University Vienna/ZET – Centre for Alternative and Complementary Methods to Animal Testing, Vienna, Austria; [2] Department of Orthopaedic Surgery, Medical University Vienna/ZET – Centre for Alternative and Complementary Methods to Animal Testing, Vienna, Austria; [3] ZET – Centre for Alternative and Complementary Methods to Animal Testing, Vienna, Austria; [4] Core Unit for Biomedical Research, Medical University Vienna, Vienna, Austria; [5] BioMed – Society for the Promotion of Biomedical and Medical Technological Research in Upper Austria, Linz, Austria

The HET-CAM (Hen Egg Test-Chorioallantoic Membrane) angiogenesis test system was standardised for validation by use of a head-mounted operating microscope for both experimental procedures and digital documentation. Today HET-CAM test procedures, documentation and interpretation are usually performed macroscopically with a common hand-held camera. This modus makes it difficult to recognise fine structures and risks generation of artefacts due to bleeding, membrane rips, contamination with bacteria/yeasts and cooling the *in vivo* test system below the critical incubation temperature of 37°C. Reproducibility of generated data is therefore often unsatisfactory.

Head-mounted microscopy systems offer improved resolution and can also be used in class I/II safety cabinets, minimising the risk of contamination and permitting testing of substances potentially hazardous for the staff. To establish a Standard Operating Procedure (SOP), CAM dissection and specimen application procedure protocols as well as the technical equipment must be standardised. Miniature head-mounted operating microscopes like the Varioscopeâ M5 tested by the authors enables the user to analyse objects difficult to access with the required magnification and operating distance and to manipulate them precisely. Automatic sensors detect the object continuously and adjust the optics, documentation is digitally performed from the experimentor's visual angle, zoom is stagelessly variable, the pivoting radius is 72°. This kind of optical vision enhancement is used in operating theatres/dental clinics and manufacture/quality control of precision components. Varioscopeâ M5 allows standardisation of CAM experimental procedures following Good Laboratory Practice (GLP) rules.

Lecture

Standardisation in cell and tissue culture – the need for specific GLP guidelines in the cell culture laboratory (Good Cell Culture Practice - GCCP)

Gerhard Gstraunthaler
Department of Physiology and Medical Physics, Innsbruck Medical University, Innsbruck, Austria

The cultivation of eukaryotic cells has become a powerful technique in basic cell and molecular biological research, applied biotechnology, and *in vitro* alternatives. Before cell culture could be carried out successfully, two problems had to be overcome: (1) Populations of cells had to be established from single cells; and (2) these populations had to be maintained for many generations. In a successful propagation of cells *in vitro*, cells from various tissues should grow and proliferate under appropriate culture conditions, while preserving highly differentiated functions, which closely resemble their ancestor cells *in vivo*. Thus, cell proliferation and cell differentiation are two major, albeit opposing, end points in tissue culture. Which of these contrasting goals should be achieved depends on the aim of a selected cell culture study and thus, on the culture conditions applied: (i) the supplementation of culture media with growth factors or differentiation factors, (ii) the use of specific extracellular matrix components, (iii) the subcultivation intervals and seeding densities, (iv) the feeding cycles, and (v) stationary cultures versus dynamic media supply in perfusion reactors. In sum, a number of tissue culture parameters have to be defined and coordinated. However, despite the widespread use and broad applications of cell and tissue cultures, a significant number of basic questions and methodological protocols are still unsolved and are handled in various ways by tissue culture laboratories. Selected examples will be presented, on how culture medium composition, medium volumes, feeding cycles, serum supplementation, or use of extracellular matrix components will influence growth of cultured cells and the expression of differentiated functions, which represents a serious impact on the credibility, reliability, reproducibility, and comparability of *in vitro* alternatives.

In conclusion, a minimum set of standards has to be defined in order to establish reproducibility and interlaboratory comparability of results obtained with *in vitro* cell culture technologies. Therefore, in analogy to GLP, a Good Cell Culture Practice (GCCP), i.e. good laboratory practice in the cell culture laboratory, was initiated at the 3rd World Congress on Alternatives and Animal Use in the Life Sciences in Bologna, 1999. As a result, GCCP Guidelines were elaborated by an ECVAM Task Force and published 2002 in *ATLA 30*, 407-414. Following this publication, a new GCCP Task Force was convened at ECVAM, Ispra, Italy, in order to produce an updated GCCP Guidance document (*ATLA 33*, 261-287, 2005), which will be presented in an accompanying lecture.

Lecture

The reference in *in vitro* studies: Quality assurance and assessment

Sebastian Hoffmann
ECVAM, Institute for Health and Consumer Protection, Joint Research Centre, European Commission, Ispra, Italy

To assess the relevance of any kind of test, it is usually linked to a reference by comparing the results obtained with it to those of a reference standard (test). Regarding *in vitro* tests, usually the routine test, i.e. most often an *in vivo* test, constitutes this reference standard. For the comparison, the reference standard results of substances to be tested in the alternative test are collected from various sources, i.e. retrospectively. Indeed, the availability of reference data is a critical and often limiting factor for the chemical selection. However, quality aspects of the *in vivo* data can thus often not be controlled. But as their quality might have a tremendous impact on the relevance assessment of the *in vitro* test, it is crucial to assure its evaluation. For example, if reference data are searched for, this search should be structured, complete and unbiased. In any case, the obtained reference data should be documented including all relevant quality information. For example, chemical identity, chemical properties, GLP-compliance, guideline-compliance, selected doses or number of animals is important information indicating quality. In the compilation and documentation of this information, the completeness and transparency, allowing a complete quality assessment, is most important. Once retrieved, the data should be inserted into databases simplifying their evaluation. Furthermore, chemicals with high quality data should be chosen to build training and calibration sets for the setup and performance checks of *in vitro* tests in order to assure their results' quality.

Lecture

Long term reproducibility of EpiOcular™, a 3-dimensional tissue culture model of the human corneal epithelium

Mitchell Klausner, Patrick Hayden, Joseph Kubilus and John Sheasgreen
MatTek Corporation, R&D, Ashland, USA

The EpiOcular tissue model (OCL-200) is an organotypic model of the human corneal epithelium cultured from normal human keratinocytes. Since commercial introduction in 1995, personal care and household product companies have increasingly used EpiOcular to determine the ocular irritancy of their products without using animals. Currently, validation of the EpiOcular model as a replacement for the Draize rabbit eye test is underway in the US. In addition, a validation study sponsored by ECVAM is scheduled to begin in 2005.

For commercial and regulatory purposes, a model must be reproducible within a given lot and between lots, especially over extended periods. Regulators and end users need to be assured that these test methods will provide consistent, good quality data during the validation process and over time.

Quality control of weekly lots of EpiOcular is performed using the MTT assay, which historically has been the *in vitro* endpoint of choice for European and US regulators. The exposure time needed to reduce the viability to 50% (ET_{50}) for a positive control is determined.

Yearly average ET_{50} values have ranged from 20.6 minutes (2000) to 25.0 minutes (1998). The coefficients of variation (CV) for tissue exposed to the negative control (ultrapure H_2O) have averaged under 6% and the average CV for all tissues has never exceeded 6.5%.

These results over the past 8 years of commercial production show EpiOcular to be a highly reproducible, stable toxicological model that is ideally suited for industrial and regulatory ocular irritancy studies.

Poster

Long term reproducibility of EpiDerm™, an epidermal model for dermal testing and research

Mitchell Klausner, Patrick Hayden, Joseph Kubilus and John Sheasgreen
MatTek Corporation, R&D, Ashland, USA

An *in vitro* model of human epidermis, EpiDerm (EPI-200), cultured from normal human epidermal keratinocytes has been sold by MatTek Corporation since 1993. Weekly lots of EpiDerm are produced for dermal irritancy, product efficacy, percutaneous absorption, pharmacological, and basic skin research studies.

In 2000 and 2002, respectively, European and US regulators approved the use of EpiDerm to assess the skin corrosivity of chemicals. Validation studies utilising EpiDerm for phototoxicity and skin irritation are currently underway.

For commercial and regulatory purposes, models must be reproducible within a given lot and between lots, especially over extended periods. Regulators and end users need assurance that the *in vitro* models will provide consistent, good quality data during the validation process and over time.

To address tissue reproducibility, quality control (QC) testing of each EpiDerm lot involves both a positive (1% Triton X-100) and a negative control (water). Using the MTT assay, which historically has been the endpoint of choice for European and US regulators, a dose response curve is constructed and the exposure time that reduces the tissue viability to 50% (ET_{50}) is interpolated.

The yearly average ET_{50} since 1996 has varied from 6.2 hr (2003) to 7.5 hr (1998). The coefficients of variation (CV) for the negative control averaged under 7%; the average CV for all tissues has never exceeded 12%.

Over the past 10+ years of commercial production, EpiDerm has remained a highly reproducible, stable toxicological model that is ideally suited for industrial and regulatory toxicology and other skin related studies.

Lecture

Ensuring quality of *in vitro* alternative test methods

Amy S. Rispin [1] *and K. Stitzel* [2]
[1] U.S. Environmental Protection Agency, Office of Pesticide Programs, Washington D.C., USA;
[2] Consultant, West Chester, Ohio, USA

In vitro and *ex vivo* methods have been developed or are under development to reduce or replace animal usage in toxicity tests. Consensus is developing in the scientific community for the quality control measures needed for *in vitro* methods; including appropriate controls, data reporting elements, and benchmarks to be identified in test guidelines so that the potential risks of chemicals can be reviewed and reliably assessed. Consistent with the goal of obtaining scientifically sound test data for hazard and risk assessment of chemicals, changes have been made in current policies and procedures to facilitate the acceptance of data developed using these methods. National and international organisations have developed policies and standards for scientific practice to assure quality in implementation of *in vitro* methods. ICCVAM and ECVAM have developed the Performance Standards process to allow proprietary test systems using *in vitro/ex vivo* methods to be accepted for regulatory use, where Performance Standards include use of reference chemicals, essential test method components and statistical performance results. Additional guidance has been provided for OECD's Good Laboratory Practice principles which will help to ensure that *in vitro* tests used for regulatory purposes are reproducible, credible, and acceptable. Generic test guidelines incorporating Performance Standards are being written to allow acceptance of proprietary test methods by regulatory agencies and to provide assurance that any *in vitro* system performs over time in a manner that is consistent with the test system as it was originally validated. Future developments should address standardised data reporting elements for special techniques such as cell and tissue culture or microarrays.

Poster

The HET-CAM assay as model to determine and compare several pharmacological activities of natural compounds without using animals

Jürgen Schneele and Jürgen Reichling
University of Heidelberg, Institute of Pharmacy and Molekular Biotechnology, Dep. Biology, Heidelberg, Germany

Many people prefer natural drugs instead of synthetic because of their lower side-effect risk. Often the therapeutic use of natural drugs is based on traditional knowledge, never proven by modern scientific methods. We decided to evaluate the HET-CAM assay, utilising the well vascularised chorioallantois membrane (CAM) of fertile hen' eggs. This assay is useful to detect a number of important pharmacological actions close to the *in vivo* situation.

The HET-CAM assay was evaluated regarding irritative, anti-inflammatory and anti-angiogenic effects of several common essential oils. The irritative activity was determined by identifying the "irritation threshold". Anti-inflammatory potential was detected ranking the phenomenons "star like vascularisation" and "granuloma formation", the supposed endpoints of inflammation. Anti-angiogenic action was identified by detecting a vessel free area around a drug containing carrier.

Detecting the irritation potential was very successful. It was possible to rank essential oils by their irritation threshold. Evaluating the HET-CAM assay as an anti-inflammatory test system by ranking the noted endpoints failed. No reproducible results could be obtained, even for the established anti-inflammatory drug hydrocortisone. It also wasn´t possible to identify any other specific endpoint for inflammation inhibition on the CAM. The studies to evaluate the anti-angiogenic HET-CAM assay have just started. First results indicate that creating a vessel free area around the carrier is possible. We decided to determine this action by a "yes/no" decision. We will further evaluate the HET-CAM assay to serve as a detection model for several pharmacological activities of different natural compounds without using animal tests.

Poster

Applying Good Laboratory Practices (GLPs) to *in vitro* studies, one laboratory's perspective

Amanda Ulrey, Rodger Curren, Hans Raabe, Greg Mun and John Harbell
Institute for In Vitro Sciences, Inc., Gaithersburg, MD, USA

The steady increase in industry use and regulatory acceptance of *in vitro* test methods has resulted in an increased need to apply Good Laboratory Practice (GLP) regulations to these systems. The original GLP regulations, developed to address the conduct of animal studies, are concerned with many special conditions that apply to animal housing and care, and the relatively long duration of animal studies that are not present in the shorter *in vitro* studies. In animal studies, for example, emphasis is placed on the isolation of species and periodic analysis of feed and water, whereas in non-animal studies there is increased importance on the justification of the test system. Recently the OECD has published advisories (No. 7, The Application of the GLP Principles to Short-term Studies, 1999; No. 14 The Application of the Principles of GLP to in vitro Studies, 2004) to clarify the application of the GLP principles to both short term and *in vitro* studies. This poster outlines the approach applied at the Institute for In Vitro Sciences, Inc. (IIVS) to the conduct of *in vitro* GLP-compliant studies. We describe the translation of the OECD guidance documents into a framework for conducting assays which use *ex vivo* tissues, monolayer cell cultures, reconstructed skin constructs, and manufactured test kits. We are grateful to auditors from numerous study sponsors and regulatory agencies who have helped us develop what we feel is a best practices approach.