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A Novel Quantitative In Vitro Model of Angiogenesis

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Summary
Quantification of angiogenesis, which is done particularly in animal models, is a prerequisite for the determination of the angiogenic or angiostatic effect of substances. In the frame of this study, a new method for quantification of angiogenesis in vitro was established based on staging of angiogenesis by breaking down the angiogenic cascade into quantifiable steps. This method allows quantification of all phases of angiogenesis up to the development of lumenised, capillary-like structures in vitro. Validation of the method showed that routine and reproducible performance should be possible for different investigators with a maintainable effort of time and costs.

Keywords: angiogenesis, quantification, in vitro model, replacement

Introduction

The process of angiogenesis includes sprouting of new vessels from pre-existing ones, their remodelling and regression. Angiogenesis is a pre-requisite for growth and differentiation of organs and tissues as it allows for supply with nutrients and oxygen (Folkman, 2003). Angiogenesis also plays a key role in many pathological processes, particularly growth and metastasis of tumours (Folkman, 1995; Caldwell et al., 2005; Banning, 2005). Both, the stimulation of angiogenesis to generate new vessels in ischemia and the inhibition of angiogenesis, so called antiangiogenesis, to arrest growth and metastasis of tumours, are promising therapeutical concepts (Augustin, 2003).

Angiogenesis occurs in a characteristic multi-step cascade of migration, proliferation, differentiation and three-dimensional organisation of endothelial cells to generate new vessels with an internal lumen. Angiogenesis as well as antiangiogenesis are regulated by soluble factors, which may influence endothelial cells in the different stages of the angiogenic cascade (Augustin, 2003).

Quantification of angiogenesis and antiangiogenesis, i.e. their depiction in measurable factors, is a basic requirement for determination of angiogenic or angiostatic effects of substances. Most quantitative investigations are undertaken in animal models. The most frequently used models are the cornea model, the chorioallantoismembrane (CAM)-model and several skin preparations (Hasan et al., 2004; Bahramsoltani, 2004). It cannot be obviated that these investigations are associated with pain for the animals. Up to now some two- and three-dimensional in vitro models of angiogenesis have been developed (for example: Montesano et al., 1983; Nehls and Drenckhahn, 1995; Hoying and Williams, 1996; Meyer et al., 1997; Peters et al., 2002; Rookmaaker et al., 2005; Wang et al., 2005; Pozzi et al., 2005). However, in these models, the effects of the substances tested were quantified in only a few phases of angiogenesis.

The aim of this study was to establish a method to quantify angiogenesis in vitro in order to achieve a replacement and complementary method comprising all stages of the angiogenic cascade. Furthermore, routine accomplishment of quantification should be possible for different investigators with a maintainable effort of time and costs.
**Materials and methods**

Microvascular endothelial cells used were isolated from *corpora lutea* of slaughtered cattle in different development stages (Plendl et al., 1996; Plendl, 1997; Plendl, 2000). Endothelial cells were identified by endothelial markers including the localisation of von Willebrand factor, vascular endothelial growth factor receptors 1 and 2 (VEGFR-1 and 2) and platelet/endothelial cell adhesion molecule (PECAM) (Plendl et al., 1996; Plendl et al., 2002a,b).

Cultivation of endothelial cells was carried out on gelatine (1.5% in PBS, Difco Laboratories, Detroit, USA) coated 24 well plates (Iwaki, Tokyo, Japan). Cells were seeded in a concentration of 41,000 to 45,000 cells per well.

Basic medium included Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% foetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin (10,000 U/ml) (all from Sigma-Aldrich, Taufkirchen, Germany).

Endothelial cells were stimulated for angiogenesis using a selective medium, which additionally contained 10% foetal bovine serum, 1% BME-vitamins (Sigma-Aldrich), 1% heparin-solution (0.25%; Sigma-Aldrich), 2% Endothelial Cell Growth Supplement (2.5 mg/ml; Schubert & Weiss, Munich, Germany) und 20% S 180-conditioned medium (see below) in basic medium.

S 180-conditioned medium was produced by cultivation of murine sarcoma cells (courtesy of Prof. R. Auerbach, University of Wisconsin-Madison, WI, USA) in basic medium. Medium was removed and spun down with 1050 U. Supernatant was sterile filtered with 0.2 µm filters (Schleicher & Schüll GmbH, Dassel, Germany) and deep frozen at -20°C until use.

For quantification of angiogenesis digital pictures of defined areas were taken with a video camera (Inteq 000610; Inteq, Berlin, Germany) using the image editing system Axiovision (Version 3.0; Zeiss, Jena, Germany) twice a week. Visual fields were standardised by always taking pictures at the same magnification (100x) and of the same area (691,200 µm²).

**Results**

For quantification of all phases of angiogenesis the angiogenic cascade was broken down into quantifiable steps.

Time dependent changes of endothelial cell morphology in the course of the angiogenic cascade were classified into 8 defined stages:

- **Stage 1**: Confluent monolayer
  - Cells in cobble-stone pattern
- **Stage 2**: Endothelial sprouting, early phase
  - Sprouting in < 50% of cells
- **Stage 3**: Endothelial sprouting, late phase
  - Sprouting in > 50% of cells
- **Stage 4**: Linear side-by-side arrangement of cells, early phase
  - Linear arrangement in < 50% of cells
- **Stage 5**: Linear side by side arrangement of cells, late phase
  - Linear arrangement in > 50% of cells
- **Stage 6**: Networking of endothelial cells
  - Network of linearly arranged cells (fig. 1)
- **Stage 7**: Three-dimensional organisation of cells, early phase
  - Appearance of lumenised capillary-like structures; verification of an internal lumen by electron microscopy (fig. 2). Capillary-like structures are defined as linear structures of endothelial cells with a diameter of more than 28 µm.
- **Stage 8**: Three-dimensional organisation of cells, late phase
  - All linearly arranged cells form lumenised capillary-like structures

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**Fig. 1**: Endothelial cells from bovine corpus luteum after 40 days in culture. Network of linearly arranged cells. Phase contrast microscopy, 100x.

**Fig. 2**: Endothelial cells from bovine corpus luteum after 42 days in culture. Longitudinal section of a capillary-like structure with an internal lumen. Transmission electron microscopy, 3,200x.
Quantification of angiogenesis was carried out by evaluation of the stages over the time course.

For verification of reproducibility this method of quantification of angiogenesis was validated. Validation included staging of angiogenesis by different persons and analysis of variability of angiogenesis over time in different culture dishes.

**Staging of angiogenesis by different persons**

Endothelial cells were incubated with selective medium and observed by phase contrast microscopy over 60 days. After a confluent monolayer was formed, visual fields were chosen randomly and documented twice a week (17 days).

These 68 pictures of cell images were evaluated in random order by two different and independent investigators. Evaluation of cell images depended on the defined stages of angiogenesis in vitro. The criterion for the choice of investigators was their comparable knowledge and experience in cultivation and microscopic evaluation of endothelial cells. Each cell image was assigned to a defined stage of angiogenesis. Subsequently, the difference between the stages assigned by the two investigators was calculated for each visual field on each day of investigation. These differences varied in a range between 0 and 1 and, except for two days of investigation, the sum of differences for each day of investigation was $\leq 1$.

Additionally, the difference of assigned stages was investigated over the total time (17 days of investigation). For this, the sum of values assigned over 17 days for each visual field and each investigator was calculated ($S$). Differences in these sums ($\Delta S$) ranged between 0 and 5 (fig. 3) with an arithmetic mean of $\Delta S = 2.8$. Assessment of stages of angiogenesis by different investigators resulted in an inter-person deviation (PD) of 4.1%:

$$HID (4.1\%) = \frac{MVD (2.8)}{MVS (6.81)} \times 100$$

**Variability of the course of angiogenesis in different culture dishes:**

Endothelial cells were seeded in 12 culture wells and cultivated with selective medium. On the first day of investigation six visual fields were chosen randomly in each well and photographed. Three visual fields were chosen in the border area and three visual fields in the centre of the culture well. Thus, investigations were carried out in 72 visual fields, which were defined by coordinates and documented twice a week.

For semiquantitative analysis of angiogenesis in different wells of the culture dishes on each day of investigation, cell images of these 72 visual fields were assigned to one of the defined stages of in vitro angiogenesis (1-8).

Documentation of visual fields and thus semiquantitative analysis was carried out over 19 days of investigation.

For each visual field the time dependent course of angiogenesis was investigated by calculating the sum of stages of angiogenesis in vitro ($S$) over all 19 days of investigation. In all 72 visual fields $S$ ranged between 81 and 102, whereas both the minimum (81, slowest course of angiogenesis) and the maximum (102, fastest course of angiogenesis) appeared in the same well.

Homogeneity of the course of angiogenesis in each well was evaluated by calculating variance ($s^2$) and deviation ($s$) of the sum of stages ($S$) of the six visual fields.

Variances of wells ranged between 9.8 and 80.6, its arithmetic mean amounted to $s^2 = 22.1$.

This resulted in deviations between 3.1 and 9.0 with a middle deviation of 4.7. Median of variances amounted to 16.6 leading to a deviation of 4.1.

![Fig. 3: Staging of angiogenesis by different investigators. Sum of stages of angiogenesis within a period of 60 days in 4 visual fields. Black: Investigator 1; Grey: Investigator 2.](image)
Excepting the well with the highest variance, a middle variance of $s^2 = 16.8$ and a middle deviation of 4.1 were calculated. Variability of angiogenesis over the course of time in the different culture wells was verified by analysis of variance components (Sachs, 1993) within the culture wells (22.1) and between the culture wells (10.3).

**Discussion and conclusions**

This report describes the establishment and validation of a new method to quantify angiogenesis in vitro. Compared to other in vivo and in vitro models of angiogenesis this method allows quantification of all phases of angiogenesis by breaking down the angiogenic cascade into quantifiable steps (stages of angiogenesis 1 to 8).

Assessment of stages by different investigators was different in only 19.1% of pictures. These differences always occurred in transition periods between stages and amounted to 1 stage only. Both investigators assigned the stages in the pictures in the correct chronological order starting with stage 1 to stage 8. Thus, results show that quantification of angiogenesis in vitro can be carried out by different investigators within the setting of one experiment.

Variability of the course of angiogenesis in vitro in different culture dishes was investigated in twelve culture wells. Calculation of variations resulted in deviations of 3.1 to 9.0. However, the deviation of 9.0 diverged widely from the others and was found in the culture well with the highest (102) and lowest (81) sum of stages. When this well was excluded, calculation of the middle variation resulted in $s^2 = 16.8$ with a middle deviation of $s = 4.1$. Compared to the median of deviation (4.1) of all 12 wells it becomes apparent that the wide divergence in this one well was not representative. Thus, results indicate a justifiable variability of the course of angiogenesis in vitro in eleven of twelve culture wells.

Analysis of variance components (Sachs, 1993) showed that the variation between the culture wells was lower than within the culture wells. Thus, the number of culture wells used could be reduced. Further calculations showed that representative quantification of angiogenesis can be carried out in a sample size of four culture wells with the examination of four visual fields per well.

Consequently the in vitro model presented allows a viable quantification of angiogenesis in vitro based on continuous observation of all stages of the angiogenic cascade. So far this was possible only in a few in vivo models of angiogenesis, i.e. the cornea model, the CAM-model and in the dorsal skinfold chamber.

Quantification of angiogenesis in these in vivo models however is limited by inflammation induced angiogenesis resulting from insertion of the angiogenic stimulus (Auerbach et al., 2003). Further disadvantages of the in vivo models of angiogenesis are the high number of animals required and the high effort of time and costs (Kruger et al., 2001; Donovan et al., 2001). Validation of the in vitro method established results in a small sample size which is time and cost efficient.

Depending on surrounding tissue cells, a high variation is found for angiogenesis in vivo (Campochiaro and Hackett, 2003). Thus, conclusions drawn from analysing in vivo models of angiogenesis are often less significant than assumed. Therefore, one advantage of our and other in vitro models of angiogenesis, which mostly are based on monocultures of endothelial cells, is the absence of the influence of other cells and tissues.

In all in vivo models quantification of angiogenesis is based on the ascertainment of the number of vessels (Auerbach et al., 2003). However, angiogenesis is defined as the process of vessel generation (Folkman, 2003). Thus, in vivo models of angiogenesis only allow quantification of the products of angiogenesis (Bahramsoltani and Plendl, 2004). The new in vitro method allows both investigation of molecular mechanisms and their relation to the stages of angiogenesis (Haas, 2005; Ribatti and Ponzoni, 2005; Manson et al., 2005).

Application of angiogenesis stimulators and inhibitors for the treatment of many diseases is in the focus of modern research (Gowda et al., 2005; Markkanen et al., 2005; Huber et al., 2005; Gasparini et al., 2005). Therefore numerous animal experiments are carried out in this promising field.

The newly established and validated in vitro method to quantify angiogenesis can be employed either in trial studies of potential angiogenic and angio-static substances in vitro, respectively, or in the investigation of their cellular mechanisms. It may provide an efficient method to replace animal testing, particularly in preclinical screening of new angiogenic or angio-static substances.

**References**


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The Importance of Good Cell Culture Practice (GCCP)

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Summary

Following a suggestion made at the 3rd World Congress on Alternatives and Animal Use in the Life Sciences (1999) and the subsequent publication in 2002 of outline guidelines on Good Cell Culture Practice (GCCP), a second ECVAM Task Force was convened, 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 has been published in ATLA in 2005 and is being made freely available, is based on six operational principles, which are briefly summarised in this paper.

Keywords: best practice, cell culture, guidance

Introduction

The use of in vitro systems is likely to expand dramatically in the future, not only in basic research, but also: to meet regulatory requirements for chemicals and products of various kinds; in the manufacture of various biological products; in medical diagnostics; and in therapeutic applications such as tissue engineering, and cell and gene therapy. Further significant developments are certain to result from the use of in vitro systems for high throughput screening in pharmacology and toxicology; the human genome project; the emerging fields of genomics, proteomics and metabolomics; and the use of biomarkers of disease, susceptibility, exposure and effect. Bearing this in mind, and because 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, it was proposed in 1999, at a workshop held during the 3rd World Congress on Alternatives and Animal Use in the Life Sciences (Hartung and Gstraunthaler, 2000), that guidelines should be developed to define minimum standards in cell and tissue culture, to be called Good Cell Culture Practice (GCCP). This proposal was endorsed by the Bologna Congress participants as a whole (Hartung et al., 2000).

Later that year, an ECVAM Task Force on GCCP was established, which produced outline guidance in 2002 (Hartung et al., 2002). A second ECVAM Task Force was convened in 2003, with a broader range of expertise in cell and tissue culture, in order to produce a more-detailed GCCP guidance document, which could be of practical use in the laboratory. Its report, “Guidance on Good Cell Culture Practice”, was published in ATLA in 2005 (Coecke et al., 2005), and is being made freely available by various routes.

The aim of this Guidance is to encourage the maintenance of best practice and to reduce uncertainty in the development and application of animal and human cell and tissue culture procedures and products, by encouraging greater international harmonisation, rationalisation and standardisation of laboratory practices, quality control systems, safety procedures, recording and reporting, and compliance with laws, regulations and ethical principles. The scope of the document has deliberately been broadly defined, to include systems based on cells and tissues obtained from humans and animals, and issues related to the characterisation and maintenance of essential characteristics, as well as quality assurance, recording and reporting, safety, education and training, and ethics.

The Guidance is intended to promote high standards in all aspects of the use of cells and tissues in vitro, and to complement, but not to replace, any existing guidance, guidelines or regulations, including the guidance on Good Laboratory Practice and Good Manufacturing Practice.

Outline of the GCCP Guidance

The Guidance is based on six operational principles, the basis of which will be briefly summarised.
Establishment and maintenance of a sufficient understanding of the in vitro system and of the relevant factors which could affect it

This principle is concerned with the essentials of assuring reliability and accuracy when using cell and tissue-based systems, namely: authenticity (including the identity of the system, its provenance, and confirmation of genotypic and/or phenotypic characteristics); purity (freedom from contamination); stability; and functional integrity. The standardisation of in vitro systems begins with the original animal or human donor, then the cells or tissues derived and their subsequent manipulation, maintenance and preservation.

The Guidance therefore deals with: isolated organs or tissues, primary cultures and early passage cultures, and cell lines; in vitro culture conditions (media, sera, additives, antibiotics); handling and maintenance (temperature, atmosphere, pH); cell detachment and subculture; cryopreservation; microbial and viral contamination; and cellular cross-contamination.

Assurance of the quality of all materials and methods, maintain the integrity, validity, and reproducibility of any work conducted

This principle is focused on quality issues related to ensuring the consistency, traceability, and reproducibility of in vitro work with cells and tissues. Each laboratory should have designated persons responsible for overseeing the quality assurance of: cells and tissues; media and other materials; methods, protocols and SOPs; equipment and its maintenance; recording procedures; and expression of results. Some of these matters are the responsibility of suppliers, rather than end users, and this may involve international standards, such as European Norms, ISO standards or pharmacopoeial protocols.

In addition to the procedures for cells and tissues and other materials, appropriate procedures are needed for items such as low temperature storage systems, incubators, gas cylinders and pressure regulators, laminar air flow and safety cabinets, automatic pipettes and pipettors, sterilisation ovens and autoclaves, and analytical and production equipment.

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

As in any practical science, clear documentation of the systems used and procedures followed is mandatory, in order to permit the traceability, interpretation and repetition of the work. This documentation should include details of: the objective of the work; the rationale of the approach; the materials, equipment, protocols and SOPs used; the origin and characterisation of cells and tissues used; cell and tissue preservation and storage; and the laboratory records, including results, raw data and quality control data.

The format of a report on the work will depend on the target audience, e.g. in-house personnel, a client or sponsor, a regulatory body, the scientific community, or the general public. The person(s) responsible for the report should be identified. Where appropriate, the report should be formally authorised for its intended purpose. A high-quality scientific report should cover the objective of the work, the protocols and SOPs used, planning and experimental design, the execution of the study, data collection and analysis, and a discussion of the outcome.

It should also be made clear that the whole study was established and performed in accordance with any relevant standards, regulations, statutes, guidelines or guidance documents, and safety and quality assurance procedures. A statement of compliance with the GCP principles should also be included.

The Guidance suggests that, when a report on cell or tissue culture work is submitted for publication, journal editors should require a minimum set of information, which should cover the origins of the cells or tissues, their characterisation, maintenance and handling, and the procedures used. Examples are given to illustrate how this requirement might be applied in the case of a cell line (mouse 3T3 cells) and in the case of primary/early passage human cell cultures.

Establishment and maintenance of adequate measures to protect individuals and the environment from any potential hazards

National and local laws govern safety in the workplace in most countries. Many countries also issue guidelines on occupational health and laboratory safety, and individual laboratories may also have rules which reflect local circumstances.

The Guidance on safety in the cell culture laboratory in no way replaces these laws and regulations, but highlights issues specific to the in vitro culture of animal and human cells and tissues. In many countries, each laboratory is required to appoint a “biological safety officer”, and this individual should be engaged in the safety evaluation of any cell culture procedures.

In the laboratory, where hazards may be complex and their evaluation requires specialist knowledge, risk assessment should be performed in a structured way. Furthermore, the results of such risk assessments should be recorded, not only to confirm that they have been carried out and appropriate action taken, but also to act as a reference document for individuals performing the tasks assessed. These assessments should be reviewed at regular intervals, to take into account any changes in local practice, national or international regulations, or increases in scientific knowledge.

Particular attention should be paid to risks which may be specific to, or more significant in, certain groups of workers. For example, where there is the possibility that women of reproductive age would be at greater risk from the effects of certain chemicals, such as teratogens or biological agents, and where persons have a diminished immune response.

The safety conditions highlighted relate not only to the safety of individual cell and tissue culture workers, but also to that of their colleagues, the general public and the environment.

All personnel must be made aware of the potential hazards associated with their work, and must be trained in the designated safety procedures, as well as in the appropriate use of the safety
equipment required (including personal protective equipment) and the appropriate handling of spillages.

The risk assessment should include the following: facilities (e.g. laboratories, offices, storage and sanitation), security, especially where special security precautions required, health and safety of staff, laboratory equipment, infectious/biohazardous materials, chemicals and radioactive substances, hazard prevention, transport, and waste disposal.

**Compliance with relevant laws and regulations, and with ethical principles**

From an ethical and legal point of view, it is desirable that high standards for cell and tissue culture should be established and maintained worldwide, so that accountability, safety and ethical acceptability can be universally guaranteed, insofar as that it is reasonably practicable. The ethical and associated legal issues raised are extremely complex and beyond the scope of the Guidance. However, all concerned should maintain a sufficient level of awareness of the ethical issues related to cell and tissue culture work, of public opinion, and of the relevant legislation at the national and international levels.

At present, there are no ethical guidelines relating specifically to general cell culture practices, but various guidelines, regulations and laws are in place for dealing with cells and tissues of specific origin and/or use.

From a general perspective, diligence in legal and ethical matters leads to data of higher value, since it can help to avoid waste of effort and encourage greater confidence in the outcome of the study, to the benefit of all concerned, including the general public. The more specific considerations include the ethical implications of: using material of animal origin (in the light of the Three Rs, and including concerns about endangered species, monoclonal antibodies, the use of fetal cells or tissues, the collection of serum [especially fetal bovine serum], and the pre-treatment of animals [e.g. to induce CYP450 enzymes]); using material of human origin (origin, use of fetal material, consent, confidentiality, ownership, patents, safety); transplantation, regenerative medicine, and stem cell research and therapy; and genetic manipulation (including the creation, storage, transport, use and disposal of genetically engineered cells).

**Provision of relevant and adequate education and training for all personnel, to promote high quality work and safety**

The range of applications for cell culture is expanding rapidly and involves an ever-broadening range of technical manipulations for use in basic and applied science, manufacturing, diagnosis, and efficacy and safety testing procedures, as well as for providing therapeutic materials.

The competence of staff to perform their duties is central to ensuring that work is performed according to the standards of the organisation in relation to its scientific, legal and safety requirements and obligations. This requires education and training, as well as the regular monitoring of performance.

A good basic education should be given in the nature and purposes of cell and tissue culture, which is an essential basis for any future training programme. The basic principles of in vitro work, aseptic technique, cell and tissue handling, quality assurance, and ethics, should be included. It is also important that those working with material of animal or human origin should have a sufficient understanding of any additional laws or regulations that will apply to them.

Much of the training required may best be given on a one-to-one basis in the laboratory. However, there are a number of principles that can be covered in organised courses that may involve participants from more than one laboratory.

Training should be seen as an ongoing process for improving and developing practical skills and maintaining competence. Given its critical importance, there should be a formally documented training programme for all members of staff, including training records and regular reviews of training needs. To ensure the quality of work in the long term, it is also important to link training with personal development programmes for technical and scientific staff, to ensure they are progressively trained and educated in line with changing laboratory activities and demands.

When new staff join a laboratory, their skills and experience should be assessed, and the need for specific further training in relation to their new jobs should be identified.

Training can be provided in-house by experienced members of staff and/or visiting experts, via accredited on-line programmes and/or through attendance at external courses. For certain applications, including product manufacture and testing, and the processing of cells and tissues for clinical use, training must be formally recorded and reviewed.

**Concluding remarks**

An electronic version of the Guidance is freely available for use in any ways which serve the interests of GCCP. Its authors hope that it will be widely used to establish and maintain best practice in all aspects of cell and tissue culture work, not only by those who are responsible for such work, but also by those who publish its outcomes or who make important policy decisions based on the information it provides.

These proceedings also contain further comments on the need for GCCP guidelines, by one of the members of the ECVAM Task Force, illustrated by some specific examples (Gstraunthaler, 2006).

**References**


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Standardisation in Cell and Tissue Culture
The Need for Specific GLP Guidelines in the Cell Culture Laboratory
(Good Cell Culture Practice – GCCP)

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Summary
The in vitro 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. For this purpose, the cell environment in vivo has to be mimicked in vitro. Thus, a number of selected culture conditions and methodological protocols have to be applied, which are handled quite differently in different tissue culture laboratories. Therefore, for good laboratory practice in the cell culture laboratory, well-defined and precisely described culture protocols are mandatory to ensure optimal and reproducible culture conditions, and to enable interlaboratory comparability of data and scientific results obtained with cultured cells.

Keywords: good cell culture practice (GCCP), cell culture, culture media, extracellular matrix, permeable culture supports

Introduction
Cell and tissue culture is defined as (1) the maintenance or cultivation of human or animal cells in vitro, including the culture of single cells (cell culture), or (2) the maintenance or growth of tissues in vitro, in a way that may allow differentiation and preservation of their architecture and/or function (tissue culture) (Schaeffer, 1990).

In the last decades, cell cultures have become indispensable tools in basic research and applied biomedical sciences. A number of applications of human and animal cell cultures were developed and introduced successfully: (i) to study the normal physiology and biochemistry of cells and their transformed counterparts (i.e. cancer cells), (ii) to test the effect of compounds on specific cell types, (iii) to produce artificial tissue (e.g. tissue engineering), and (iv) to synthesise valuable products (biologics) in large-scale bioreactors. This increased use of in vitro methodologies is indeed a beneficial development in terms of the 3R concept of Russel and Burch (Balls et al., 1995), decreasing the number of experimental animals.

The major advantage of using cell culture is the consistency and reproducibility of results that can be obtained using a batch of cells of a specific type, or, preferably, a homogenous clonal cell population maintained under Good Laboratory Practice (GLP)-based culture protocols.

Human and animal cell culture is rooted in two fundamental concepts in biology. On the one hand on the cellular concept of Schwann and Schleiden in the early 19th century, postulating the cell as the fundamental unit of life, and on the other hand on the concept of homeostasis, the constant maintenance of the internal milieu within tissues, organs, and organisms (McKeehan et al., 1990).

For successful growth and maintenance of human or animal cells in vitro, either primary cultures or continuous cell lines, appropriate culture conditions are required that optimally mimic the physiological conditions (internal milieu) in vivo et situ (Gstraunthaler, 2003). Thus, the microenvironment of a cell has to be established in vitro and be provided by the culture system: (i) temperature, (ii) extracellular ion milieu and osmolality, (iii) extracellular pH and buffering, (iv) basal supply with essential nutrients and oxygen, (v) supplementation with growth factors and hormones, (vi) culture substrates and growth supports, and (vii) disposal of metabolic end products.

As can be expected, a number of variables can be introduced into cell and tissue culture: (i) the supplementation of culture medium with growth factors or differentiation factors, (ii) the use of appropriate culture substrates and/or specific extracellular matrix components, (iii) the subcultivation intervals and seeding densities, (iv) the feeding cycles, and (v) stationary cultures versus dynamic medium supply in perfusion reactors. All in all, 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 different ways by cell culturists.

In this brief overview, selected examples will be presented on how culture medium composition, medium volume, feeding cycle, serum supplementation, or use of extracellular matrix components will influence growth of cultured cells and the expression of differentiated functions, which represents a seri-
ous impact on the credibility, reliability, reproducibility, and comparability of in vitro alternatives.

**Culture medium**

The culture medium is one of the most important single factors in cell and tissue culture. The culture medium must supply all essential nutrients for cell metabolism, growth and proliferation (Barnes et al., 1987; Butler and Jenkins, 1989; Ham and McKeehan, 1979). These include biosynthetic precursors for cell anabolism, catabolic substrates for energy metabolism, vitamins and trace elements whose function is primarily catalytic, and bulk inorganic ions (electrolytes) whose functions are both catalytic and physiological, e.g. to maintain culture medium pH and osmolality within acceptable limits.

Normal osmolality of human extracellular fluids (plasma and interstitium) is about 290 mosmol/kg. Thus, it is reasonable to assume that this is the optimum osmolality for human and mammalian cells in vitro. Certain variations have to be taken into account for other species (e.g. amphibia, insects).

In the renal medulla, however, an interstitial osmolality of up to 1200 mosmol/kg is generated by the countercurrent system of the kidney, providing the basis for the urinary concentrating mechanism in antidiuresis for salt, water, and volume homeostasis. When cultured renal cells of distal tubule, collecting duct, or papillary origin, like MDCK and PAP-HT25 cells, were adapted to hypertonic culture conditions by adding NaCl, raffinose, or urea to the culture medium, the cells responded with the accumulation of organic osmolytes as seen in renal medullary and papillary cells in vivo (Handler and Kwon, 1993). This strategy of “hypertonic stress” in culture enabled renal physiologists to study the cellular and molecular mechanisms of renal medullary osmoadaptation in vitro (Burg, 1995; Handler and Kwon, 2001).

In the nephron, proximal tubular epithelial cells are capable of gluconeogenesis from C3-precurors, like lactate, pyruvate or alanine. The application of “metabolic stress” by culturing LLC-PK1 and OK proximal tubule-like renal cell lines under glucose-free culture conditions resulted in the selection of gluconeogenic substrains, termed LLC-PK1-FBPase+ and OKGNG+, respectively (Gstraunthaler and Handler, 1987; Gstraunthaler et al., 1993). Both cell lines express the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and fructose-1,6-bisphosphatase (FBPase). Furthermore, LLC-PK1-FBPase+ cells respond to metabolic acidosis, i.e. a decrease in culture medium pH from 7.4 to 6.9, with adaptive increases in PEPCK activity and enhanced glutamine metabolism (Gstraunthaler et al., 2000; Holcomb et al., 1995), making it a valuable in vitro model to study renal acid-base adaptation and pH-mediated gene expression at the cellular and molecular level (Curthoys and Gstraunthaler, 2001).

**Oxygenation and culture medium volumes**

Human and animal cells are characterised by oxidative metabolism. Thus, a sufficient supply of cultured cells with oxygen is critical to satisfy cell metabolism. In small cultures (e.g. T-flasks, culture dishes), the oxygen demand can be met by gas diffusion from the headspace through the culture surface. Under normal culture conditions, the availability of oxygen to cells growing under 3-6 mm of medium is adequate.

When renal proximal tubular cells are brought into tissue culture, they revert from oxidative metabolism and gluconeogenesis to high rates of glycolysis. Among the factors possibly responsible for this metabolic conversion, limited oxygen availability and/or substrate supply are discussed. In order to study the role of these factors in long-term cultures, the impact of culture medium volume, growth conditions, culture medium volume, and glucose content on carbohydrate metabolism of the continuous renal cell lines LLC-PK1 (porcine kidney) and OK (opossum kidney) was investigated (Gstraunthaler et al., 1999).

The impact of both culture medium volume and glucose content was determined by overlaying confluent monolayer cultures of LLC-PK1 and OK cells (i) with increasing volumes of culture medium, thereby increasing the amount of glucose and increasing the diffusion distance for oxygen, and (ii) with increasing culture medium volumes at constant absolute amounts of glucose by adding glucose-free medium, in order to increase volume and thus decrease oxygen supply at a constant glucose supply. Alternatively, and in order to improve cell oxygenation, LLC-PK1 cells were also cultured in roller bottles. Cell carbohydrate metabolism was assessed by measuring rates of glucose consumption and lactate production, respectively, and by determination of specific activities of the key glycolytic enzymes hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK), and lactate dehydrogenase (LDH). Mitochondrial phosphate-dependent glutaminase (PDG) was assayed as marker enzyme for oxidative metabolism of glutamine.

In LLC-PK1 and OK cells, rates of glucose consumption were independent of the initial glucose concentrations and/or the culture medium volumes used. Glucose was quantitatively converted to lactate, which accumulated in a 1:2 molar ratio. Lactate in the culture medium reached a maximum content after 24 h and was re-utilised by the cell lines thereafter. Interestingly, the rates of lactate re-uptake strictly depended on culture medium volume, indicating a volume-induced stimulation of oxidative lactate metabolism. Marked changes were found for the specific activities of glycolytic enzymes. In LLC-PK1 cells, increased glucose supply caused increases in HK, PFK, PK and LDH activities, which were superimposed on the stimulatory effects of increased medium volumes. Enzyme activity showed a biphasic response, indicating that both glucose supply and culture medium volume covering the cell monolayer, and thus oxygen availability, are factors determining glycolytic rates of LLC-PK1 renal cells. As expected, under conditions of enhanced oxygenation of LLC-PK1 cells in roller bottle culture, glycolytic enzyme activities decreased, whereas PDG activity increased, which was paralleled by increased rates of ammonia generation. Thus, changes in nutrient supply and oxygenation of renal epithelial cell cultures by altered culture medium volumes dramatically influence metabolic rates and levels of enzyme activities (Gstraunthaler et al., 1999).
Culture substrate and cell adhesion

Most cultured cells, primary cultures as well as continuous cell lines, are substrate- or anchorage-dependent, which means that the cells require attachment to a given surface (culture substrate) in order to survive, proliferate, and express their differentiated functions (Ruoslahti and Öbrink, 1996; Balda and Matter, 2003). In routine cell culture this is mostly accomplished by the use of surface-charged, hydrophilic polystyrene culture plastic ware, that allows crosslinkage of cells via glycoproteins and/or divalent cations (Ca²⁺, Mg²⁺). In special cases, culture vessel surfaces can be coated with components of the extracellular matrix, like collagen type IV, laminin or fibronectin to allow attachment via specific cell receptors. Also, collagen type I, gelatin or poly-D-lysine are used as surface coatings to improve adhesion and differentiation of cultured cells (Genestie et al., 1997; Gumbiner, 1996; Kleinman et al., 1987).

When MDCK renal epithelial cells were cultured on a complex extracellular matrix, i.e. basement membrane gel extracted from Engelbreth-Holm swarm tumour, the cells differentiated into tall epithelia, columnar in shape, with an ultrastructure characteristic for fluid-transporting renal epithelia (Zuk et al., 1989).

Culture dish vs. permeable filter supports

Traditionally, cultured cells are grown on impermeable culture substrates. Under these conditions, cultured renal epithelial cells form monolayers of highly differentiated and polarised cells. The cells grow with their basolateral surface on the bottom of the culture dish and thus, the apical surface faces the culture medium. Under these conditions, cultured epithelial cells can form domes, i.e. fluid-filled blisters generated by the transepithelial vectorial transport of fluid and solutes trapped between the cell layer and the water-impermeable culture dish (Gstraunthaler, 1988). However, access to growth medium on the basolateral side, at which nutrient exchange normally occurs, is restricted. In contrast, permeable, microporous membrane substrates provide independent access to both the apical and the basolateral side of the cultured epithelium, and thus offer a more physiologically relevant environment (Handler et al., 1984).

The apical and the basolateral fluid compartments are separated by the cultured epithelium, which allows determination of electrophysiological and transport parameters in Ussing-type chamber devices. It has been shown in a number of studies that the degree of differentiation considerably increases when the epithelia are cultured on permeable surfaces. Also, remarkable differences in cell shape and cell density were observed in filter-grown epithelia (Genestie et al., 1997; Gstraunthaler et al., 2000). A6 cells, for example, derived from the kidney of Xenopus laevis, respond to vasopressin with an increased rate of sodium transport only when cultured on permeable filter supports. Functional assays revealed that A6 cells do not express vasopressin receptors until an ordered epithelium has been formed on permeable culture supports (Lang et al., 1986).

This technique of culturing transporting epithelia represents the prerequisite for studying epithelial dysfunction in in vitro nephrotoxicity testing (Gstraunthaler et al., 1990; Steinmassl et al., 1995).

In conclusion, a number of tissue culture parameters substantially influence the expression of specific morphological features and cellular functions and thus the degree of differentiation of cultured cells. 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. In analogy to Good Laboratory Practice (GLP), a Good Cell Culture Practice (GCCP) should be implemented in tissue culture work. GCCP guidelines were recently elaborated by two ECVAM Task Forces (Balls et al., these proceedings; Coecke et al., 2005; Hartung et al., 2002).

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Ensuring Quality of In Vitro Alternative Test Methods

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Summary
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 on 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 assessed reliably. 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 the 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.

Keywords: in vitro, quality, performance standards, toxicology, testing, cell culture, alternatives, proprietary test methods

Introduction*

OECD members as well as other countries have legislated mandatory submission of credible scientific data for use in assessing the hazards and potential risks of chemicals to humans, wildlife, and the environment. A basic principle in such legislation is that evaluations of the safety of new chemicals must be based on toxicology test data of sufficient quality, rigor, and reproducibility. Although in vivo tests in laboratory animals have formed the foundation of hazard and risk assessment, regulatory programmes are now beginning to implement non-animal methods such as cell and tissue culture systems, and high throughput methods such as toxicogenomics and proteomics. These alternatives to current methods often refine, reduce, or replace animal use while providing a comparable or better level of protection of human health or the environment.

Test methods destined for regulatory use evolve in a systematic fashion from research and development through test method design and validation (see fig. 1).

Specific in vitro/ex vivo and other non-animal methods have been developed or are currently under development to replace animal tests (ECVAM, 2002) or to allow direct assessment of chemical effects in human cells or tissue components. Areas of alternative assay development are outlined in figure 2. When any new test method is developed, test parameters are standardised so that laboratories can obtain consistent results. Although quality assurance is always an issue, in vitro test systems pose different issues regarding their quality and performance than commonly used animal methods (Rispin et al., 2004).

Some in vitro assays include a bioconstruct or ex vivo component that acts as the target tissue for the toxicological effect of concern. The bioconstruct can be a cellular, non-cellular, or tissue construct. Tissue constructs, often made of materials derived from humans, are designed to model the toxicology of cells or tissues and replicate the in vivo responses to chemical exposure. All of the elements of the assay function together for purposes of the assay’s use to fulfil regulatory testing requirements. The quality of the bioconstruct and associated reagents must be assured for any assay to be used for regulated studies. Quality control ensures that the results of the assay can be reliably used in hazard and risk assessment and can be compared with data from previous studies within a laboratory and between one laboratory and another. Because these systems can be affected by small changes in method or components, such test systems must be well defined and function reproducibly.

Once a new method is developed, it must be validated (OECD, 2005). Validation involves systematic laboratory studies performed on a set of common reference chemicals to determine the new test’s reliability in terms of intra- and inter-laboratory variability, and to assess how well it functions for various chemical classes.

*Much of this material has been previously published in Regulatory Toxicology and Pharmacology (Gupta et al., 2005; Rispin et al., 2005 (in press)).
In the United States, the Interagency Coordinating Committee for Validation of Alternative Methods (ICCVAM) provides for review and assessment of the validity of the new toxicology tests including non-animal alternative test systems and proprietary test methods (ICCVAM, 2003). The European Centre for Validation of Alternative Methods (ECVAM) has been established to facilitate development of non-animal tests for the European Union and to assess the reliability and relevance of such tests for European regulatory mandates (ECVAM, 1995). Both ICCVAM and ECVAM are directed to seek alternative tests which reduce, refine, or replace animal testing.

Once a new *in vitro* method is validated and accepted for regulatory use, companies and regulatory authorities making decisions based on the data need assurance that it will continue to perform in a manner consistent with the test system as it was originally validated. Stability of performance of the *in vitro* system must be ensured over time, particularly if there are changes in components of the test system, changes in test system manufacturer(s), and/or changes in the prediction model. Testing laboratories must use good scientific practice, as well as appropriate calibration and standardisation methodology established by the various technical disciplines appropriate to the elements of their assay system.

**Special considerations for proprietary test methods**

Additional issues arise when the *in vitro* methods are developed, validated and registered by manufacturers for commercial marketing as proprietary test methods. Generally, the process for acceptance of such proprietary test methods calls for special approaches. At OECD, the specific proprietary test system cannot be accepted under the Mutual Acceptance of Data Decision, but this OECD agreement does allow for use of a generic guideline based on the proprietary test method. This allows other companies to enter the marketplace with “me-too” methods; a “me-too” method is mechanistically and functionally similar to a validated method. Regulatory acceptance of these generic methods is based on validation data obtained using the proprietary version. The European Union and OECD use generic guidelines for proprietary test methods, following their validation. If a proprietary test method assay system is identified by a United States agency for use in regulatory testing, the agency cannot mandate use of the specific proprietary test method, but can develop Performance Standards for a generic protocol incorporating the salient features of the proprietary test method such that a competitor may introduce a product that meets the Performance Standards. A United States agency may then state that the proprietary test method meets the Performance Standards. The applicant for approval of a chemical who wishes to use a different proprietary test method may do so as long as documentation is provided to the agency that the manufacturer of the second proprietary test method has assured compliance with the Performance Standards (USEPA, 2003, 2004). OECD has issued three generic guidelines which incorporate elements of Performance Standards for *in vitro* assessment of dermal corrosivity: EpiDerm™/EPISKIN™ – OECD 431; Corrositex® – OECD 435; and Transcutaneous Electrical Resistance Test (TER) – OECD 430.

Once a new proprietary test method is accepted for regulatory use, both regulatory agencies and the users of these test systems need a process to ensure that “me-too” test kits developed according to the generic descriptions produce results similar to those obtained using the system originally validated and accepted.

**Quality assurance for regulatory use**

International quality control requirements for assays performed to fulfil regulatory requirements are called for under the OECD Mutual Acceptance of Data Decision which sets forth international standards for Good Laboratory Practice (GLP) (OECD, 1981). The principles of GLP have been developed to promote the quality and relevance of test data used for determining the safety of chemicals and chemical products. GLPs apply to non-clinical health and environmental safety studies for the registration of pharmaceuticals, veterinary drugs, pesticides, and food...
and feed additives, and for the regulation of industrial chemicals, cosmetic products and consumer products.

GLPs are concerned with the organisational process and the conditions under which non-clinical health and environmental safety studies are planned, performed, recorded, archived and reported. Their aim is to assure consistency, traceability and reproducibility of test results. Test facilities are required to follow GLP principles when carrying out studies to be submitted to national authorities for the purpose of assessment of chemicals and other uses related to the protection of man and the environment. GLP principles are accepted throughout OECD countries and world-wide (USEPA, 2002; USFDA, 2003). Within countries, GLPs are implemented by regulatory authorities.

GLP and good science responsibilities of the testing laboratories include using a pre-defined study protocol for the assay, training the technical staff and ensuring that staff skills are maintained, employing controls and calibrated equipment, employing established and defined regulatory acceptability criteria for the test method, and accurately and completely documenting each step in the procedure. GLP principles apply to in vitro tests as well as tests using animals. Previously GLP guidelines focused primarily on in vivo methods and required significant interpretation to cover in vitro methods (Cooper-Hannan et al., 1999).

Policy framework to assure quality of alternative methods

When an alternative test method matures, its regulatory use depends on availability of a comprehensive scientific and policy framework. Due to the efforts of ICCVAM, ECVAM, OECD, scientific societies, and the animal welfare community, a six element framework is now in place internationally to assure quality of in vitro alternative methods: OECD Process for Proprietary Test Methods; Performance Standards – ICCVAM; Application of the principles of GLP to computerised systems; Application of the principles of GLP to in vitro studies; and Guidance on Good Cell Culture Practice (GCCP) (see fig. 3) (Gupta et al., 2005; Rispin et al., 2005).

OECD consensus document on application of GLP to in vitro studies

Some time ago, OECD developed special guidance for application of GLPs to computerised systems (OECD, 1995). More recently, recognising that in vitro methods will be more prominent as alternatives or supplements to traditional in vivo safety testing, ICCVAM and ECVAM recommended to OECD that it develop special guidance for the application of GLP to in vitro studies. The new OECD Advisory Document for in vitro studies (OECD, 2004) calls for study directors to document that in vitro test methods be validated, or are structurally, functionally and mechanistically similar to a validated reference test method and have comparable performance. In addition, representative batches of test components should be evaluated for their performance.

Performance Standards can provide a framework for application of GLP to in vitro assays. (See details in Performance Standards section below.)

The OECD advisory document recognises the growing use of cell/tissue culture and anticipates developments in toxicogenomics, toxico proteomics, toxico metabolomics and microarrays and is expected to facilitate interpretation of GLP for in vitro studies performed for regulatory purposes.

ECVAM task force on good cell culture practice (GCCP)

In 1999, based on development of several promising cell culture test systems as alternatives to animal tests, the Third World Congress proposed that cell culture procedures be standardised to meet a number of testing and regulatory needs (see fig. 4). The first four principles of GCCP apply equally well to microarrays, biochemical assays, or computational methods developed as alternative methods. ECVAM has issued two reports on GCCP (Hartung et al., 2003; Coecke et al., 2005), which specifies procedures to ensure that test systems are free of any contamination or other diseases or conditions at the beginning of the study that might interfere with the outcome of the study and calls for the origin (species/tissue), source, arrival condition and mainte-
formance requirements to be documented and confirmed at the laboratory on a regular basis. These GCCP reports are intended to set standards for education and training in Europe, to educate the editors of journals to look at the critical cell culture parameters when a paper is submitted, and to help regulatory authorities in the acceptance and interpretation of in vitro data.

Scientific societies and other organisations are also developing standardised data reporting elements or quality assurance measures for other promising alternative technologies such as microarrays, software systems, etc.

**Performance Standards**

The elements of Performance Standards are summarised in figure 5. Although originally planned for regulatory acceptance of Proprietary Test Methods, they provide a comprehensive approach for specifying performance of any alternative test methods. Performance Standards are descriptive and functional and serve to demonstrate that any kit or assay system is mechanistically and functionally equivalent to the test that was initially accepted for regulatory purposes (ICCVAM, 2003). Reference chemicals drawn from the data base used for validation of the original proprietary test method are used to establish functional criteria for each test method (ICCVAM, 2003).

**The use of additional controls when using in vitro methods**

The role of experimental controls (positive, negative, or graded) is normally spelled out in agencies’ policies for acceptability of test procedures. In recent years, the consistent quality of the test animals as well as concerns for animal welfare have persuaded many authorities to do without concurrent positive controls for most acute tests involving laboratory animals. However, for alternative methods in which animals are not involved, laboratories should use positive as well as negative controls and benchmark chemicals (defined below) as part of every chemical trial using in vitro assays. The use of appropriate controls ensures lot-to-lot consistency of the biological or ex vivo assay components of proprietary test methods as well as non-proprietary tests and verifies that test systems are calibrated and functioning properly. Such chemicals may be selected from the Performance Standard reference chemicals for the in vitro assay.

The assay endpoint value(s) for the negative control should fall within an acceptable range as determined by historical experience with the test system. The negative control test system units, e.g. tissue constructs, are manipulated in parallel with those treated with test and positive control materials. The assay endpoint is measured on these test system units and provides a baseline value for comparison with values from the test system elements treated with test and positive control materials. This baseline may be the maximum viability value or the absence of lesions in a histological evaluation. In viability assays, the negative control value provides the “100% viability” value for determination of the relative viability of the test substance-treated tissues.

The positive control material is used to assess the functional characteristics of the test system and the execution of the assay. The positive control-treated test system units are tested in parallel with the negative control and test material-treated units each time the assay is performed. Positive controls should be chosen so that the test system response falls in the mid range of the pos-

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**Fig. 5: Performance Standards for proprietary test methods and other validated in vitro alternative methods**

- OECD Process for Proprietary Test Methods → Generic guidelines and reference chemicals
- Application of the principles of GLP to computerized systems → OECD Consensus Document Monograph 10, 1995
- Application of the principles of GLP to in vitro studies → OECD Advisory Document Monograph 14, 2004
- Guidance on Good Cell Culture Practice (GCCP) → ECVAM Task Force on GCCP – 2002, 2005
sible responses and both increases and decreases in response against the historical performance of the positive control can be detected. The results from the control assays help to confirm that a valid trial was performed when test data for each unknown chemical are submitted to regulatory agencies. In addition, trend analysis of control trials can be used to detect drift in the assay system.

Benchmark chemicals with well-characterised responses selected from the same structural or functional chemical class as the unknown or test chemical provide assurance that the test is performing correctly for the specific class of compound being tested. A benchmark chemical may be included in each experimental trial along with the positive and negative controls.

The use of concurrent controls is critical to ensure that testing laboratories and regulatory authorities can be confident that in vitro results continue to fulfil their intended regulatory function. In addition, properly selected controls can ensure that proprietary test methods are functioning as designed. Toxicologists must be provided with appropriate standardisation or verification approaches, including appropriate controls, to ensure that proprietary assay systems are functioning properly.

**Responsibilities for ensuring quality data from in vitro methods**

Practically speaking, quality control of in vitro assay systems must be a shared responsibility of the manufacturer of the proprietary test method or supplier of tissues or tissue constructs, the contract laboratory that uses the assay system, and the company that submits the test for regulatory acceptance. Bioconstructs or cell cultures may have a short shelf life. Proprietary test methods and other in vitro systems may be relatively expensive; therefore, the number of replicate systems available for quality control efforts by testing laboratories may be limited by practical considerations. In light of these concerns, the user may be dependent on the manufacturer for many of the basic elements of quality control, including cell or tissue characterisation and functional performance of the assay system. The manufacturer should be expected to provide adequate documentation of quality control testing of representative assay systems for each manufactured batch. In addition, the user must provide a quality control check in the laboratory on a regular basis appropriate to the test system so that the assay materials can be shown to perform as expected after transport and handling. Regulatory authorities will need to develop submission guidelines that are clear and consistent with the realities of the new regulatory test systems.

**Summary**

Ensuring quality of results from in vitro and ex vivo test systems in regulatory toxicology testing calls for innovative approaches by all concerned; testing laboratories, regulatory authorities, validation organisations, and the scientific community. In preference to imposing new regulations, existing statutes, directives and implementing regulations are being interpreted to extend their guidance to the many new technologies used to replace or augment traditional animal tests.

When the validity of a new in vitro test is reviewed, the reviewing organisation normally considers use of controls and benchmark chemicals and can also recommend Performance Standards. Test system sponsors may wish to propose a set of Performance Standards for use in assuring consistency of the test system response when they prepare the new system for review of its validation status. Performance Standards required for proprietary test methods for use by regulatory agencies can also be part of the foundation for quality control of the proprietary test methods.

With experience, the expectations for quality assurance for non-animal testing will evolve. It will be important for test developers, laboratories performing the tests, toxicologists and regulatory agencies to continue to work together to ensure that guidelines meet the needs of all parties.

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