Workshop 5.11
Mechanisms of chemically-induced ocular injury and recovery

Ocular Toxicology *In Vitro* – Cell Based Assays

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Summary
Interactions between the three cell types in the cornea control differentiation and responses to stimuli. We have sequentially added cell types in a 3-dimensional construct to assess the minimal requirements for a toxicology model of the human cornea. Co-cultured cell types displayed patterns of cytokines different from the single cell-type 3D models. Following exposure to toxicants there were marked changes in cytokine profiles, related to the toxicant and markedly influenced by the epithelial cell-line used. For a rational choice of cell lines, their steady-state patterns of signal molecules should be compared to those in normal human preocular fluid.

Keywords: cornea, cytokines, human, in vitro, toxicology

Introduction and questions

The health of the ocular surface is intimately linked with quality of vision, and is also a determining factor in the success of surgical procedures. The ocular surface encompasses the outer structures of the eye (cornea, conjunctiva, and lids), the lacrimal gland and tear film and the innervation and immune structures that link them (Stern et al., 2004). Interactions within and between these components maintain the barrier to the environment and modulate responses to external stimuli (Cook et al., 2001; Stramer et al., 2003; Wilson et al., 2003; Hazlett, 2004; Holan et al., 2004; Wilson et al., 2004; Lema and Duran, 2005; Narayanan et al., 2005).

The Colipa Strategy for the Development of *in vitro* Alternative Methods focuses on physiological processes – from organ level to gene activation – that can be used to monitor responses to toxicants and recovery from injury. Among the programme aims, central to the replacement R of the 3Rs and to cell based assays, is identifying quantitative endpoints predictive of the nature and severity of injury. The ultimate aim is to replace the Draize test – currently the gold standard of ocular toxicity testing – with a scientifically valid animal-free strategy.

We needed to establish the minimal degree of complexity which is necessary and sufficient to represent the human cornea in cell culture models of toxicity. We have assessed the behaviour of undisturbed cultures and the response to a single toxicant application in constructs from monolayers to three-dimensional cultures comprising stratified human corneal epithelia and quiescent human stromal cells, to probe stability and correlates to injury and recovery.

Starting from the premise that depth (cell types) and area are the defining characteristics of an injury (Jester et al., 1998a; Jester et al., 1998b; Jester et al., 2000; Jester et al., 2001), we have explored endpoints related to metabolism and barrier function and sought correlations with patterns of cytokines, mediators that are known to be secreted by, and influence, the different cell types of the ocular surface. For the refinement R of the 3Rs and, more importantly, to anchor the *in vitro* tests with *in vivo* physiology, the add-on benefit of the latter endpoints is that signal molecules are readily detectable in (human) tears, and available through non-invasive tests.

Materials and methods

Epithelium

In addition to availability and stability with passage number, minimum *a priori* criteria were set for choosing human corneal

or conjunctival epithelial cell lines: ability to stratify and establish a barrier to penetration. Acute toxicity is correlated with barrier function (Konsoula and Barile, 2005). The corneal stroma is protected by the tight barrier of epithelium, which is in turn protected by the preocular fluid. A number of corneal and conjunctival epithelial cell lines were assessed for stratification, morphological differentiation and trans-epithelial electrical resistance (TEER).

Initially, epithelia were cultured according to originator's instructions: the cultures were then transferred and maintained in fully defined medium without antibiotics (KGM Bulletkit, Cambrex Biosciences, NJ, USA). Stratification was achieved by supplementing the medium with 1.25 mM $CaCl_2$ and cultured at liquid-air interface. The choice of serum-free medium follows from the need for minimally/non-activated stromal cells. This medium will not interfere with toxic effects and endpoint measurements.

Stroma

With approval of the Committee for Ethics in Research and consent for use in research, donor corneas unsuitable for transplantation were used to generate primary human corneal fibroblasts. These were amplified in DMEM with 10% foetal bovine serum (Invitrogen, Paisley, UK) and cryopreserved. Cells used to populate a collagen type I gel were maintained for at least 1 week in fully defined medium to inhibit cell proliferation and activated phenotype. Before seeding in collagen gels the cells were dissociated using trypsin. Soybean trypsin inhibitor (Sigma, Poole, UK) was used to stop the enzyme.

The matrix was built using 3 mg/ml bovine skin type I collagen (Vitrogen, Angiotech Biomaterials, Ca., USA), gelling at pH 7.4 (Taliana et al., 2000). Cells were included in the collagen solution at a concentration of 6 x 10^4 cells/ml. The gels, volume 0.25 ml per 24 well insert, were cast at their final placement and maintained in fully defined medium for 2 weeks before any further manipulation.

Epithelial-stromal construct

At least 2 weeks after matrix formation, epithelial cells were seeded at 10^5 cells/gel. The construct was maintained for 2 days and then lifted to liquid-air interface, where it was cultured for a further 7 days.

Toxicants and exposures

The choice of toxicants unifies the Colipa project: they are all from the same batch and are distributed to the collaborating laboratories by Colipa. We tested NaOH (Riedel-de-Haën, Germany), Sodium dodecyl sulphate (SLS, OmniPur®, Merck, Germany), and the non-ionic surfactant Tomadol 45-7 (Tomah, Ca. USA), each prepared in tissue culture water (Sigma). The concentration chosen was 0.66%, at which NaOH produces an initial lesion covering approximately three quarters of the surface of a stratified epithelium. For monolayers, this concentration was halved to take into account the smaller mass of the construct.

All constructs were exposed to 10 µl toxicant applied topically for 10 minutes and followed by an exhaustive wash with 1.0 ml tissue culture medium. Cultures were followed at 1 and 4 h for early signs of injury and then at 1, 3, 5, and 7 days post-exposure to evaluate the ensuing dynamics of responses to injury and recovery.

Assays

Trans-epithelial electrical resistance: TEER was measured with Millicel Electrodes (Millipore, Ma., USA) held in a fixed support. A cell-free insert was included as reference at each measurement.

Protein: After solubilisation with 1% Triton X-100 (Sigma) overnight at 4ºC, protein was quantified using the bicinchoninic acid kit (BCA™ Protein Assay Kit, Pierce, IL, USA) according to manufacturer's instructions.

Alamar Blue: This reagent (AlamarBlue, Serotec, Oxford, UK) was used at 2.5%, a concentration with minimal toxicity on repeat cell loading, every other day for 14 days. Fluorescence was measured using a Spectramax fluorimeter (Molecular Devices Corporation, Ca., USA) at $\lambda_{excitation}$ = 545 nm and $\lambda_{emission}$ = 590 nm, and expressed in arbitrary units. The sensitivity of the instrument was fixed for all readings.

Cytokines: TNF, IL-1β, IL-6, IL-8, IL-10 and IL-12p70 were measured simultaneously in each supernatant sample using the BD Cytometric Bead Array (BD Biosciences, Ca., USA) on a FACSCalibur (BD Biosciences). The fluorescence of samples and standard dilutions was assessed at least twice in each assay. Results are presented as means of a number of cultures, while the mean of replicate readings counted as 1. The dynamic range of the assays were 5-5000 pg/ml for IL-6 and IL-8.

Results

Some cell lines did not fulfil the minimal criteria for inclusion in tests and were not investigated further. For example, the immortalised human corneal epithelial cell line (Canadian cells, gift from May Griffith) reached only a fraction of the TEER achieved by the immortalised human corneal epithelial cells (USA cells, donated by Ilene Gipson), as shown in figure 1. The TEER of IOBA-normal human conjunctival cells (gift from Yolanda Diebold) was equal to that of the Canadian cells. Note that *in vivo* the conjunctiva is much leakier than the cornea.

It is interesting to note that the cytokine levels were specific to each cell line: in Araki-Sasaki supernatants IL-6 and IL-8 were in the order of 30 pg/ml and 400 pg/ml respectively, while in the Canadian cells levels were around 1500 and 2000 pg/ml respectively. In contrast, the USA cells secreted no detectable IL-6 when stratified. There was a clear time dependence of cytokine secretion, strongly influenced by the culture model. Stratification and culture at liquid-air interface, each affected the pattern and secretion of cytokines.

Further changes were observed when epithelia were cultured with keratocyte-populated stroma. An advantage of using a collagen matrix that can be altered by the cells growing therein becomes evident in that shrunken collagen gels can be easily identified. The difference in cytokine secretion from shrunken and unaltered gels is an indication of interactions between stro-

mal and epithelial cells (fig. 2), and stromal and endothelial cells (cell line gift from May Griffith). IL-8 concentrations also changed in response to the above factors. No cytokines were detected in supernatants of keratocyte gels.

When epithelial and endothelial cells were co-cultured with medium separating the two cell types, we observed a larger influence on IL-6 and IL-8 cytokine secretion than when in direct contact. Concentrations and magnitude of change with two cell types also depended on the medium used.

Preliminary experiments suggested that the optimum experimentation period would commence seven days after air-lifting and that the stratified epithelium would show no signs of attrition for seven days thereafter. We compared the different constructs for the projected duration of the experiment by measuring a metabolic correlate with Alamar Blue, protein as a measure of cell proliferation, and cytokine production. For monolayer cultures the starting point was taken at confluence, judged by microscopic inspection. While there was a continuous increase in cell mass (protein concentration) in the monolayer, the absolute redox activity remained stable, suggesting a decrease per cell. Protein concentrations and reductive activity were decreased in the USA stratified epithelia after day 5 of the experiment, while the epithelial-stromal constructs were stable for 7 days. Cytokine production per unit mass increased in monolayers, while in the stratified epithelia and the epithelial-stromal constructs cytokine levels were much lower and little changed during the experiment (fig. 3). Remarkably, IL-6 production was not quantifiable in stratified epithelia, and the levels of IL-8 were also below those of constructs containing a stromal cell seeded gel and stratified epithelia.

In response to single, short-term toxicant applications all constructs showed decreases in total reductive activity and cell mass (protein concentration). However, the time course and extent of these changes varied according to the construct: the non-ionic surfactant caused a very early and devastating decrease in cell number and activity in the monolayer. These losses of cells and metabolic capability occurred later in stratified epithelia; they were smaller and preceded by a short-term increase in reductive activity in epithelial-stroma constructs. In the case of this toxicant, an increasing secretion of IL-6 and IL-8 was measured towards the end of the experiment in the epithelial-stromal construct, perhaps suggestive of some recovery.

Discussion and conclusions

In response to public demand and European legislation, testing of cosmetic ingredients on live animals will cease in the near future. A consensus has emerged that any methodology that is developed to replace the use of animals in ocular toxicity testing has to fulfil a number of *a priori* criteria in order to be considered for pre-validation. These criteria include scientific purpose, mechanistic basis, statement of limitations and appropriate controls.

Within the Colipa Strategy for Development of *in vitro* Alternative Methods programme we have aimed at paradigms that address these criteria. Damage to the cornea is the most serious in functional visual terms, hence it was chosen as the target organ representing the ocular surface. We have reasoned that mediators of intercellular communication that can also be involved in inflammation would be a mechanistically-linked correlate of the response of the cornea to external stimuli. Inflammation is an aggravating result of ocular injury, and an integrating response of the ocular surface.

The results presented here indicate that cytokine production

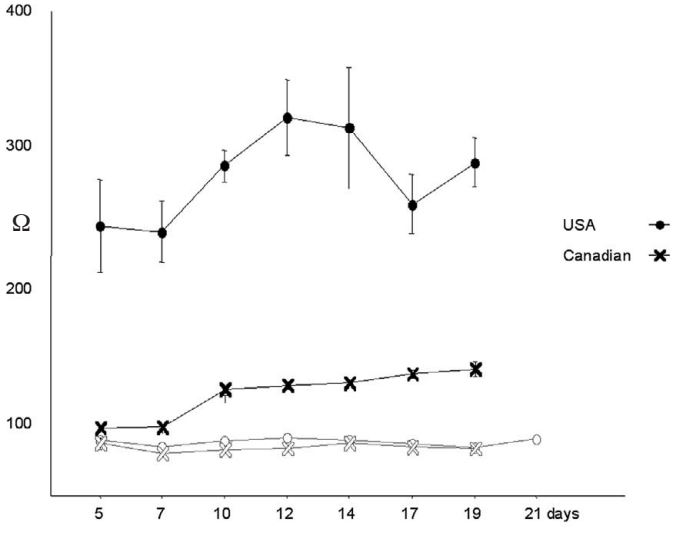

Fig. 1: Trans-epithelial electrical resistance
USA cells: filled circles; Canadian cells: filled crosses; blank inserts: grey, unfilled symbols

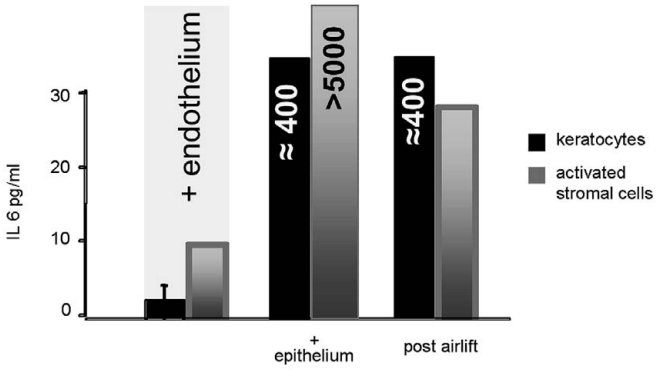

Fig. 2: IL-6 levels in different culture conditions
Black bars: quiescent keratocytes; grey bars: activated keratocytes (shrunken gel)

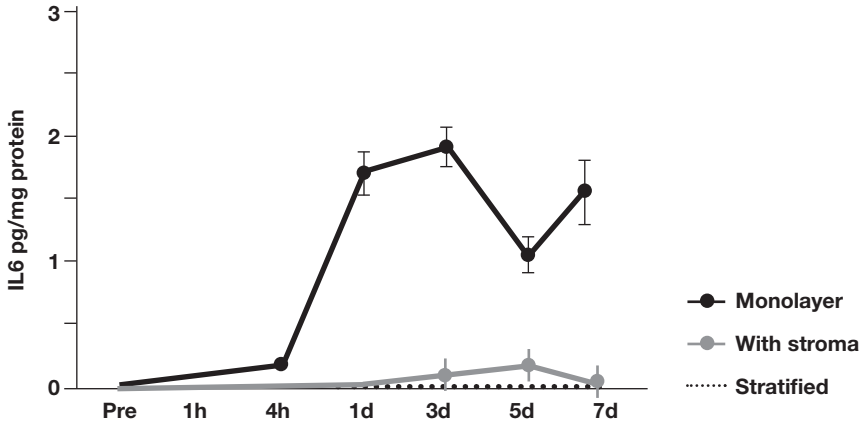

Fig. 3: IL-6 secretion in cell culture constructs with USA epithelia and quiescent keratocytes
Black line: monolayer; grey line: epithelialised stroma; dashed line: stratified epithelium

reflects the type and three-dimensional arrangement of cultured cells. Furthermore, the stability of the constructs is enhanced by the presence of different cell types. This indicates that a stratified epithelium alone is not a sufficient model for corneal toxicity tests. Elegant three-dimensional human corneal epithelial constructs, e.g. Skinethic (Van Goethem et al., 2005), could be combined with a three-dimensional stroma. Responses to a small number of chemicals suggest that the epithelial-stromal model allows some recovery to be studied and might be more discriminating than the single cell type models. In this construct, assessment of the time course of cytokine secretion after injury pointed to changes in cell activity that were not detectable by measuring reductive activity or cell mass.

There remains a caveat to the choice of cell lines in corneal constructs: each of the cell lines tested secreted different levels of cytokines. A comparison with data on cytokines in human tears is complicated by the fact that such data have not been obtained under standardised conditions, even when a healthy control group was included in the study. Our results indicate that after a chemical injury IL-6 increases in tears, compared to unexposed unrelated eyes (Berry and Jeffreys, 2001). In animals, IL-6 and IL-8 (and IL-1α) were elevated for a period of days after exposure to a toxicant, each cytokine taking a distinctive time course (Den et al., 2004). *In vitro*, twelve borderline irritation eye make-up removers increased IL-8 levels in supernatants of centrifuged CEPI monolayers from 1 to 23 times over control values (Debbasch et al., 2005), suggesting increased sensitivity of this endpoint to toxicant effects.

After sequentially building more complex corneal models and comparing their behaviour in steady state and after exposure to toxicants, we conclude that a three dimensional culture involving the three major cell types in the cornea is a stable construct and potential model for toxicity testing. Concentrations of toxicants used in these model-development studies have been at least one or two orders of magnitude smaller than those used in ex vivo studies done by our collaborators. This encourages us to further focus on mild to moderate toxicants when exploring the potential and limitations of this methodology.

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Can Toxicogenomics be Used to Identify Chemicals that Cause Ocular Injury?

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Summary

With the impending ban by the European Union on the use of the Draize eye test for cosmetic testing and to meet concerns regarding the 3Rs, an in vitro replacement is essential. This article considers a toxicogenomic approach that will allow a gene fingerprint to be produced from chemical exposure of corneal constructs prepared from human cell lines. Differential fingerprint profiles will allow identification of mild, intermediate and severe toxic preparations. This toxicogenomic approach will provide a rapid, high throughput, accurate diagnostic assay for the effects of toxic chemicals on the eye.

Keywords: Draize eye test, toxicogenomics, cornea

Introduction

The Draize eye irritation test has been mandated for routine use by manufacturers to study the safety of their products for marketing. This test, which is currently the gold standard, has several limitations:

a) It lacks objective and accurate quantification
b) It requires large numbers of animals
c) It is a slow, expensive, and a time consuming process
d) It is opposed by animal welfare groups as well as increasing numbers of the general public concerned about "non-medical" animal experimentation.

A total of 3,300 rabbits were used for eye irritation studies in the UK in 1994, of which the majority were used for cosmetics testing. It was recommended that the use of the Draize eye irritation test should be phased out within Europe by 1997, providing that suitable *in vitro* alternatives had been developed. Despite this not being the case, the European Parliament has subsequently decreed that the Draize eye test will no longer be allowed from 2009. This has left the manufacturing and cosmetic companies with a dilemma, since they must ensure safety of all new products entering the market. In an attempt to overcome these problems, alternative *in vitro* toxicity tests have been sought. Although a number of *in vitro* methods have been proposed as alternatives to the *in vivo* Draize test, they are either overcomplicated or oversimplified to such an extent that they measure only a single aspect of eye irritation (e.g. cell death) (Prinsen, 2005). Such tests are not considered sufficiently rigorous or informative, especially for moderate or mildly toxic chemicals. One possible alternative is to use a toxicogenomic approach to provide a gene fingerprint directory (i.e. pattern recognition), which will identify the toxicity of chemicals in corneal constructs. If successful, this will allow the production of specific diagnostic gene arrays for high throughput assays of all potentially toxic preparations that enter the market.

Chemical injuries to the eye

The first points of contact in chemical exposure to the eye are the eyelids, conjunctiva and cornea. Severe injury can result in corneal scarring and neovascularisation, which leads to opacification of the cornea, thus preventing light reaching the retina (Khaw et al., 2004). In extreme cases, treatment may require corneal transplantation, keratoprosthesis or limbal cell transplantation (Dogru and Tsubota, 2005). If these avenues are unsuccessful, the patient will suffer blindness or severe visual impairment. Mild chemical injury usually results in irritation and reddening. This either recovers by itself or requires some antibiotic and steroid treatment. Moderate chemicals initiate intermediate tissue damage and the outcome is dependent on the nature of the chemical (e.g. alkali, acid, detergent) and duration of exposure.

Not surprisingly, the nature of tissue damage and biological responses is very different between severe and mild chemical injury. Severe chemical injury to the cornea will result in tissue coagulation and large-scale cell death via necrosis. Immediate effects will be on the epithelium, but penetration into the stroma will cause damage to Bowman's membrane and the underlying stroma with loss of keratocytes and disorganisation of the regular collagen lamellae. This results in breakdown of barrier function, making the cornea more prone to infection, and destabilisation of corneal hydration leading to swelling. The release of cytokines and other mediators will attract macrophages, leading to an acute inflammatory response and neovascularisation. The immediate survival response is rapid proliferation of cells and disorganised matrix deposition, which often results in corneal scarring. Ulceration and corneal liquefaction can result, due to sustained upregulation of matrix metalloproteinases (MMPs). Not surprisingly, the biological responses associated with severe corneal damage result in a massive temporal change in gene expression. Furthermore, tissue remodelling can continue for up to 3 years post injury.

By contrast, mild chemical injury to the eye will result in limited tissue damage and a normal repair response. There is usually some cell loss, normally of the corneal epithelium, which is usually repaired within 72 hours through the division of resident transit amplifying cells and limbal stem cells. There will be some pain and irritation due to damage to the nerve endings in the epithelial layer. The mild associated inflammatory response will result in a reddening of the eye, which may need treatment with steroids and antibiotics. As with severe chemical injury, there will be a temporal change in gene expression, but the gene expression profile will be very different to that for severe injury.

While mild chemical injury does not normally impair vision, the discomfort to the individual is unacceptable and should be avoided if possible. Thus, it is important that manufacturers, employers and Health and Safety Executives are fully aware of potential chemical contraindications, can advise on protection and can recommend treatment following accidental exposure.

Feasible alternatives to the Draize test

Numerous alternatives to the Draize test have been proposed. These include cell culture, *ex vivo* organ culture and corneal reconstructs. While cell cultures (corneal or non-ocular, such as skin) can provide valuable information on severely toxic chemicals (the cells simply die!), they are limited in that they are usually composed of a single cell monolayer and, thus, do not measure tissue penetration. Such cultures have been used to assess the release of cytokines, growth factors and other mediators as a function of inflammation, but validation for mild or moderate chemicals is difficult due to the simplicity of the test.

Ex vivo organ culture using bovine, rabbit and porcine corneas has been attempted with outcome measures including morphology, release of biological mediators and opacification. Such models have the advantages that they have the typical 3D corneal structure, consist of numerous cell types and allow chemical penetration to be taken into account. Limitations include access to sufficient numbers of corneas, quality control (there will be considerable biological variation), the lack of a rapid throughput assay and the fact that these tissues are not human.

Corneal reconstructs (either epithelium/stroma or epithelium/stroma/endothelium) can be prepared in culture from established and well-characterised human cell lines (Griffith et al., 1999). These constructs, which produce an excellent 3D corneal architecture, allow excellent quality control and permit chemical penetration to be assessed. The limitation of these models to date is the nature of the outcome to be used to monitor mild or moderate chemical injury. Outcome measurements have tended to be similar to those described for organ culture and include morphological assessment and the measurement of the release of mediators. However, an alternative and more reliable outcome would be to monitor global gene expression changes in a construct following chemical exposure.

Aim

The strategy for the global gene expression approach is to develop a gene fingerprint directory (a pattern recognition approach), which will identify chemicals and preparations toxic to a bioengineered human cornea and that will be at least as sensitive as the Draize test.

Specific aims will include:

- Determination of different genetic fingerprints of human, bioengineered corneas exposed to different generic groups of toxic chemicals using microarray analysis.
- Recommendations for a set of selected human genes (<100) involved in eye irritation that are differentially expressed in the microarray experiments and are therefore important for the production of specific "diagnostic array" chips.

The proposed genomics assay for eye irritation will focus on pattern recognition rather than individual changes in genes. This pattern recognition approach, which depends on dynamics, dose and kinetics, will identify markers, e.g. for inflammatory processes, that may be exploited in the development of other *in vitro* assays.

Experimental design

For proof of principle, bioengineered human corneal constructs will be exposed to a range of generic chemicals (e.g. alkali, acid, detergent) with varying degrees of known ocular damage (severe, moderate, mild) and penetration, at varying concentrations, for different time periods. Affymetrix Gene Array chips will then be used to investigate changes in gene expression – using the U133A and B chips containing known and unknown genes (Wilson et al., 2002). A broad-based gene approach is essential in the first instance to avoid missing key diagnostic genes. A thorough statistical analysis using standard Affymetrix array analysis software will be undertaken and genes divided into clusters based on their functional categories. Statistically significant differences in gene expression profiles are expected for particular categories of genes under each experimental condition. Thus, it will be possible to define a gene expression fingerprint for the effect of each toxic chemical in this *in vitro* system to monitor tissue damage and repair.

Based on the gene expression profiles obtained, it is proposed to produce specific chips ("diagnostic arrays") containing selected genes that are differentially expressed in our human *in vitro* model microarray experiments. Thus, a rapid high-throughput diagnostic assay using bioengineered human corneas will be developed, which will give a global fingerprint of gene expression for the effects of toxic chemicals on the cornea. A subset of human genes will be selected that are consistently differentially expressed according to the arrays under the different experimental conditions. The possibility also exists to identify genes whose expression is consistently altered in different *in vitro* models (these genes will also be confirmed as differentially expressed

using RT-PCR and/or quantitative PCR). Individual genes and/or functional categories of genes, which are differentially expressed in all experimental paradigms, will be prioritised for inclusion on a human gene "diagnostic array", which will allow for identification of "toxicity fingerprints" in diagnostic arrays. Key diagnostic genes will include markers for inflammatory processes, apoptosis, and those for regeneration, and/or embryonic development. Selected genes will be arrayed onto glass slides for diagnostic array analysis. Appropriate array analysis software will then be used to compare the gene expression profiles obtained from diagnostic tests using cosmetics with the gene expression profiles generated by known toxic chemicals.
Of particular importance will be

1. the concentration of chemical to be tested, since potency is likely to vary significantly between *in vitro* and *in vivo*.
2. duration of exposure – minutes or hours?
3. number of exposures, should there be repeat exposures and should there be irrigation as would occur with tearing *in vivo*?
4. the timing of analysis. Since toxicity will involve both damage and repair, there will be a temporal change in the gene expression profile. Thus, the most comprehensive approach will be to look at expression profiles of early and late response genes.

Validation

The toxicogenomics model will require validation at a number of levels:

1. comparison with a database on chemical injury in humans
2. comparison with toxicity results obtained with the Draize test
3. approval by the relevant bodies (e.g. ECVAM, ICCVAM, NICEATM)

Conclusion

Toxicogenomics offers the possibility to produce a rapid, high throughput, accurate diagnostic assay for the effects of toxic chemicals on the eye. The approach, once developed and validated, will offer a high level of quality control, will be highly reliable, and will be quick and simple. Furthermore, this assay will meet the requirements of the European directive to phase out the Draize test by 2009 and should significantly reduce the number of animals used in this procedure.

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ICCVAM-NICEATM-ECVAM Symposium on Mechanisms of Chemically-Induced Ocular Injury and Recovery: Current Understanding and Knowledge Gaps

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Summary
A scientific symposium on the Mechanisms of Chemically Induced Ocular Injury and Recovery was held on May 11-12, 2005 in the USA. It was organised and co-sponsored by ICCVAM, NICEATM/NIEHS, and ECVAM, with support from COLIPA. A major goal was to identify research needed to advance the development of test systems that meet regulatory testing requirements and provide for human health protection while reducing, refining (less pain and distress), and/or replacing the use of animals. This paper (one of three), provides a symposium overview and summarises the Session 3 discussions on mechanisms and biomarkers of chemically induced ocular injury and recovery.

Keywords: ocular injury and recovery, chemically-induced, mechanisms of ocular injury and recovery, chemically-related, ICCVAM-NICEATM-ECVAM

Presentation of symposium format and results

A brief overview of the entire symposium is presented in this paper. Also summarised is the part of the meeting (Session 3) dealing with discussions of issues and research needs related to the current understanding of mechanisms of ocular injury and recovery associated with chemical exposure. Other papers by Eskes et al. and Stokes et al., describe, respectively, the other two broad areas covered by the symposium, which were: *In Vitro* Models for Ocular Injury: Current and Potential Biomarkers, and *in Vivo* Models of Ocular Injury and Recovery: Current and Potential Biomarkers to Support Development and Validation of Predictive *in Vitro* Models.

Regulatory need to understand and assess ocular toxicity potential

Accidental eye injury is the leading cause of visual impairment in the United States, and workplace and household chemicals are a significant cause of these injuries, according to sources such as the American Academy of Ophthalmology and the US National Institute for Occupational Safety and Health. US Federal agencies charged with public health protection and the regulation of chemicals or pharmaceuticals and other types of products are concerned about being able to identify potential ocular hazards. Ocular safety and hazard testing in the United States had its origins in part stemming from a well documented case from the 1930s of an eyebrow and eyelash dye that caused severe effects including blindness in women (see illustration on the following US Food and Drug Administration website: http://www.fda.gov/oc/history/historyoffda/section2.html).

Symposium overview

The two-day May 2005 Symposium on the Mechanisms of Chemically Induced Ocular Injury and Recovery was organised and co-sponsored by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), which is part of the US National Institute of Environmental Health Sciences (NIEHS), and the European Centre for the Validation of Alternative Methods (ECVAM). Additional support was provided by the European Cosmetic, Toiletry and Perfumery Association (COLIPA). The symposium was open to the public and was attended by 76 participants and speakers.

Representatives from ICCVAM, which is composed of 15 US Federal regulatory and/or research agencies, and members of the Ocular Toxicity Working Group (OTWG), one of the numerous ICCVAM subject matter working groups, assisted with symposium organisation, preparation and implementation. Liaisons from ECVAM to the OTWG also provided welcome assistance. A cadre of national and international invited experts from academia, industry, medicine, government, and the animal welfare advocacy area served as speakers and meeting co-chairs.

* The content of this paper does not represent the official position or policy of the US Environmental Protection Agency (US EPA) or any other US federal government agency

One overarching symposium aim was to review the state-of-the-science and current understanding of the pathophysiology, mechanisms and modes of action of chemically induced ocular injury, persistence and recovery. Another aim was to identify areas where research could be conducted to aid the development of test systems that would meet regulatory needs to provide for the protection of human health as well as be compatible with the promotion of animal welfare goals.

Specific symposium objectives were to:

1) Review current and potential molecular, cellular, tissue (e.g. histopathology), and clinical (e.g. corneal opacity, swelling, depth of injury, biomarkers of chemical injury and recovery and their usefulness for *in vivo* and *in vitro* testing models of ocular irritancy and corrosivity);

2) Identify knowledge gaps in the understanding of chemically induced ocular injury and recovery;

3) Identify and prioritise future research initiatives that would address current knowledge gaps and that are considered necessary to advance the development and validation of *in vitro* models of chemically induced ocular injury and recovery; and

4) Discuss and identify quantitative, objective endpoints that should be considered for inclusion in the current *in vivo* rabbit eye test and/or human clinical testing (e.g., more sensitive markers of injury and recovery) that would support development and validation of predictive *in vitro* methods and improve hazard characterisation and reliability.

The symposium agenda over the two-day period consisted of a total of five sessions. During Session 1, an overview of recent initiatives and US regulatory requirements for ocular toxicity testing was provided. Previous workshops on ocular injury and recovery were discussed, including: (1) the International Life Sciences Institute Health and Environmental Sciences Institute (ILSI/HESI) Workshop on Replacing the Draize Eye Irritation Test: Scientific Background and Research Needs, 1995, (Reference: *J. Toxicology – Cutaneous and Ocular Toxicology, 15(3)*, 211-234, 1996); (2) the ILSI/HESI Technical Committee on Alternatives to Animal Testing Expert Meeting on Eye Irritation Testing, Sept 29-30, 1996, (Reference: Ophthalmologic Perspectives on Eye Irritation Testing, Nussenblatt, et al., *J. Toxicology – Cutaneous and Ocular Toxicology 17*,103-109, 1998); (3) the COLIPA Workshop on Mechanisms of Eye Irritation, October 5-8, 1997 (Reference: Report on the COLIPA Workshop on Mechanisms of Eye Irritation, Bruner et al., *ATLA 26*, 811-820, 1998). Also mentioned was the ECVAM Workshop on Eye Irritation Testing: The Way Forward (Reference: Balls et al., *ATLA 27*, 53-77, 1998). Also noteworthy, are the results of two international workshops organised and sponsored by the [US] Interagency Regulatory Alternatives Group (IRAG) in 1991 and 1993, respectively: (1) Workshop on Updating Eye Irritation Test Methods: Proposals for Regulatory Consensus (Reference: *Food Chemical Toxicol. 31(2)*, 1993) and (2) Workshop on Eye Irritation Testing: Practical Applications of Non-Whole Animal Alternatives (Reference: *Food Chemical Toxicol. 35 (1)*, 1997).

Session 2 of the symposium reviewed current ocular injury and toxicity assessments and included a discussion of human chemically induced ocular injury. Session 3 (summarised in this paper) dealt with mechanisms and biomarkers of ocular injury and recovery. In Session 4, current *in vitro* models of ocular injury and recovery were discussed. The subject of Session 5 was *in vivo* quantitative objective endpoints to support development and validation of predictive *in vitro* models. Panel discussions were held after Sessions 3, 4 and 5. Each of the panels was given a set of pertinent questions to address based on the session topic, and the discussions were summarised at the end of the meeting.

Mechanisms and biomarkers of ocular injury and recovery

Symposium Session 3 focused on issues related to the current understanding of known mechanisms and modes of action of chemically related ocular injury, persistence and recovery. Speakers in this session discussed topics such as eye injury type and reversibility, cellular (e.g. corneal epithelium and endothelium) and tissue (e.g. cornea, conjunctiva, iris) responses to chemical injury in humans and animals, chemical-specific modes of action (e.g. for acids and bases) and the role of histopathology and depth of injury in evaluating ocular injury onset, extent, severity and recovery potential. Other speakers presented information on (1) the role of chemical toxicokinetics in ocular injury (including delayed onset of effect) and detoxification, (2) possible future roles for toxicogenomics in elucidating processes involved in ocular injury and its sequelae, and (3) the effects of chemical exposure on tear film.

Session 3 panel piscussion questions and responses

The panel for Session 3 was asked to respond to a series of four questions. Highlights of the panel's answers and opinions are presented below.

In the first question, the panel was asked to identify the currently known mechanisms and modes of action of chemically induced ocular injury and recovery. The panel responded that mechanisms of injury (e.g. cytotoxicity, protein coagulation, membrane saponification, disruption of the extracellular matrix, inflammatory cell infiltration with release of mediators, up-regulation of proteases and collagenases) are known for some chemicals and product types.

Further, the extent of ocular surface involvement and depth of corneal penetration may correlate with severity of lesions and recovery and thus could serve as a biomarker of reversibility potential. The panel proposed that more research could provide information on the general applicability of this concept. In addition, it was noted that some existing studies have shown species differences in response to the same chemical and dose-dependent differences in response to the same chemical (i.e. mechanisms of repair or injury at lower doses may not be relevant at higher doses). Currently, more data and information are available on severe ocular injury than for milder forms.

For the second question, the panel was asked to list current knowledge gaps in understanding of mechanisms and modes of

action of chemically induced ocular injuries and recovery. The panel responded that further assessment was needed of the relationship between type and severity of initial *in vivo* damage and persistence of effects. Furthermore, additional work was required to identify and develop for utility, biomarkers of injury and recovery (i.e. gene expression profiling, clustering and pathway analysis for ocular damage and repair) and to elucidate the role of tear film in ocular damage. It also would be important to improve the translation of *in vivo* physiology to *in vitro* models. This would involve enhancing current knowledge of such things as the role of metabolism and the linkage of expression of specific corneal proteins with injury and recovery processes.

In question three, the panel was asked to identify research initiatives needed to address current knowledge gaps and further characterise mechanisms and modes of action in order to advance the development and validation of predictive *in vitro* models of chemically induced ocular injury and recovery. The panel's opinion was that quantitative endpoints could be incorporated into the current *in vivo* test and evaluated to make the test more informative. These would include histopathology to correlate cellular changes with observational endpoints, HPLC and mass spectroscopy to evaluate penetration of substances into the eye, and depth of injury analysis to gain further insight on the utility of this measurement as a biomarker for reversibility/irreversibility of effects. The panel thought that further evaluation of species differences and dose-dependent differences in response was merited. Also mentioned was the need for additional work on tear film, especially with regard to composition determination, further elucidation of its role in protection of ocular constituents and the potential consequences of its disruption on the severity of effects of mild and moderate irritants. Other areas for additional work included the need to better characterise early onset versus delayed ocular responses to chemical agents, to investigate further the toxicokinetics of chemical exposure to the eye, to better elucidate the role of inflammatory responses in observed ocular damage and to better explore recovery mechanisms of the eye (such as effects on stem cells). Also suggested was additional evaluation of other *in vitro* models that might be more predictive or useful in hazard identification, such as human corneal models (isolated and reconstituted) and pig corneal models.

For the final question, the panel was asked to identify what *in vivo* biomarkers (e.g. molecular, cellular, morphological, clinical) should be further investigated as predictive indicators of severity of lesions, reversibility versus non-reversibility, or delayed responses. In answer, the panel mentioned as noteworthy: histopathology, quantitative endpoints obtained using standard biomicroscopy, confocal microscopy, selective staining, cytology, immunologic markers and gene expression profiling, and clustering and pathway analysis for ocular damage and repair.

A report of the Symposium proceedings will be available in the near future on the ICCVAM website (http://iccvam.niehs.nih.gov).

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An Overview of the COLIPA Eye Irritation Research Programme

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Summary

The COLIPA eye irritation programme incorporates research projects and collaborative activities with external partners. Three projects focus on understanding mechanisms of eye injury and identification of new in vitro endpoints that are more predictive of the in vivo human response to chemical injury: 1) investigation of whether kinetics/patterns of change in physiological function and signals of injury released from the cornea in vitro can predict a chemical's potential to damage the eye, with a focus on recovery; 2) identification of endpoints related to the magnitude of injury and quality of repair in human immortalised cells and 3D human conjunctival and corneal constructs; 3) a genomics project using a pattern recognition approach to identify new endpoints for injury/repair that builds on corneal models from projects 1 and 2. Collaboration of industry, academia, external scientific organisations and regulators is equally important. COLIPA is working with ECVAM by actively participating in its Eye Irritation Task Force and providing support for statistical analysis of current in vitro methods.

Keywords: in vitro, eye irritation, Draize eye test, alternative, replacement, COLIPA

Introduction

The efforts of the COLIPA (European Cosmetic, Toiletry and Perfumery Association) -SCAAT (Steering Committee on Alternatives to Animal Testing) Eye Irritation Task Force are dedicated to the development of *in vitro* alternative methods/strategies to assess eye irritation for the replacement of the Draize test. The research programme follows on from previous validation studies (e.g., Bagley et al., 1992; Balls et al., 1995; Brantom et al., 1997) and workshops (Bruner et al., 1998; Balls et al., 1999). The approach used incorporates integrated research projects and collaborative activities with external partners.

Research programme strategy and objectives

The approach of the COLIPA eye irritation research programme is to build on the experience of the earlier validation studies and scientific workshops through collaborations with academic institutions conducting COLIPA-supported projects and other collaborative activities with external partners. The objective is to gain an understanding of cellular molecular mechanisms of chemically induced eye irritation, with focus on corneal injury and recovery. Through this understanding the expected outcome is the identification of *in vitro* endpoints related to the dynamics of injury and recovery that are more predictive of the *in vivo* human response to chemical injury. This will enable the development of prediction models for pre-validation of new or improved *in vitro* methods that would proceed to formal validation.

There are three integrated research projects: 1) an investigation of whether kinetics/patterns of change in physiological function and signals of injury released from the cornea *in vitro* can predict a chemical's potential to damage the eye, with a focus on recovery; 2) identification of endpoints related to magnitude of injury and quality of repair in human immortalised cells and 3-dimensional human conjunctival and corneal constructs and 3) a genomics project using a pattern recognition approach to identify new endpoints for injury and repair that builds on the corneal models being evaluated in projects 1 and 2 for potential use in current/future *in vitro* assays. The approaches taken for each of these projects are given below.

Research Projects

Project 1: *In vitro* corneal culture eye irritation assay

This project is being conducted by Norbert Schrage and Markus Frenz at the University of Aachen, Germany, and was initiated in January 2002. The aims are 1) to develop an *in vitro* model of excised corneas maintained in culture to allow observation of injury and recovery following chemical exposure and 2) to investigate whether kinetics/patterns of change in physiological function and signals of injury released from the perfused cornea *in vitro* can predict a chemical's potential to damage the eye, with a focus on recovery.

The following stepwise approach has been adopted to develop a new isolated perfused corneal culture model that can be maintained for a period of time under steady culture conditions:

- Determination of viability and stability of the isolated perfused corneal culture system both morphologically and metabolically, and definition of the parameters to be used routinely to confirm system viability and stability
- Determination of suitability of the model to investigate wound healing by mechanical abrasion
- Exposure of the defined isolated perfused corneal system to model toxicants
- Identification of the morphological/biochemical markers of injury/recovery to be used routinely to evaluate toxicant effects
- Investigation of evaluation methods including biomicroscopy, pachymetry and glucose/lactate turnover for system viability/stability, LDH, cytokines (IL-1α, IL-2, IL-6 IL-8, MIP1) and growth factors (FGF, VEGF) for evaluation of dynamics of injury and recovery after mechanical trauma or toxicant exposure.

Project 2: Cell culture models for ocular toxicity studies

This project is being undertaken by Monica Berry and Marcus Radburn-Smith at the University of Bristol, UK, and was initiated in January 2002. The aims of this project are 1) to sequentially build 3D human corneal constructs consisting of epithelium, stroma and endothelium in order to better understand underlying mechanisms of action of eye irritation and 2) to identify new endpoints related to magnitude of injury and quality of repair in human corneal models that will enable prediction of the nature and severity of toxicant effects.

This following approach is therefore being used in this project to investigate the physiological responses to ocular injury (e.g. cell activation/signalling to immune system effector cells) by evaluating responses to model toxicants in increasingly complex corneal constructs:

- Investigation of human corneal and conjunctival cell lines for culture conditions, growth characteristics and suitability for use in 3D constructs
- Development of stratified epithelia, stromal equivalents and construction of two layer models (epithelium and stroma)
- Construction of three layer models by the addition of an endothelium cell layer
- Exposure of monolayers and stratified models to model toxicants using the following evaluation methods
- Light and confocal microscopy, characterisation of surface markers and differentiation, barrier formation assessment, membrane damage, metabolic activity, profiling of cytokine secretion.

Project 3: Development of gene expression fingerprints to identify toxic damage to the cornea

This project will be initiated in the summer of 2005 and will be led by Mike Boulton, University of Cardiff, U.K. The aims of this project are 1) to generate proof of concept that generic chemicals will cause differential gene expression in human bioengineered corneas 2) to identify gene expression profiles in bioengineered human corneas exposed to generic classes of chemicals and 3) to develop a gene fingerprint directory to identify chemicals toxic to a bioengineered human cornea. The principal outcome should be the application of the knowledge to better understand new endpoints for eye irritation and enable further development of current and future *in vitro* methods.

This project will use the following approach:

- Microarray analysis with human Affymetrix Gene Array chips using RNA from untreated versus treated (model toxicants) bioengineered human corneal constructs developed in the Bristol project
- Analysis expansion to define a gene expression fingerprint for the effects of each chemical class. Fingerprints will be generated for two time points to assess both injury and repair.

External collaboration

Equally important to achieve validated *in vitro* methods is collaboration between industry, academia, external scientific organisations and regulators. COLIPA is working with ECVAM by active mutual participation in both COLIPA and ECVAM Eye Irritation Task Forces to ensure that the research efforts are synergistic rather than duplicative between the two organisations. The Task Force also uses external contacts in order to benchmark and refine the eye irritation research projects.

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