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Review

Assuring consumer safety without animals

Applications for tissue engineering

Carl Westmoreland[1] and Anthony M. Holmes[2,*]

[1]Safety and Environmental Assurance Centre (SEAC); Unilever; Sharnbrook, Bedfordshire UK; [2]National Centre for the Replacement, Refinement and Reduction of Animals in Research; London, UK

Abbreviations: 3Rs, replacement, reduction, refinement; NOAEL, no observed adverse effect level; ECVAM, european centre for the validation of alternative methods; OECD, organization for economic co-ordination and development; REACH, registration, evaluation, authorization and restriction of chemicals

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Humans are exposed to a variety of chemicals in their everyday lives through interactions with the environment and through the use of consumer products. It is a basic requirement that these products are tested to assure they are safe under normal and reasonably foreseeable conditions of use. Within the European Union, the majority of tests used for generating toxicological data rely on animals. However recent changes in legislation (e.g., 7th amendment of the Cosmetics Directive and REACH) are driving researchers to develop and adopt non-animal alternative methods with which to assure human safety. Great strides have been made to this effect, but what other opportunities/technologies exist that could expedite this? Tissue engineering has increasing scope to contribute to replacing animals with scientifically robust alternatives in basic research and safety testing, but is this application of the technology being fully exploited? This review highlights how the consumer products industry is applying tissue engineering to ensure chemicals are safe for human use without using animals, and identifies areas for future development and application of the technology.

Introduction

Tissue engineering is a rapidly advancing multi-disciplinary science that brings together materials scientists, polymer chemists, bioengineers, biologists and physiologists to create living, three-dimensional tissues. The field of tissue engineering has been driven by the need to develop novel therapies for a wide range of medical needs, including relatively straightforward applications, such as using tissue engineered skin for treating burns victims to more complicated scenarios such as the much publicized recent efforts to replace a whole section of trachea.[2] Clinical application of tissue engineered products may continue to be the driving force behind further advancement in the field, but there are other opportunities for the application of the technology which are often overlooked.

Engineered tissues have increasing potential to be used as alternatives to animals for studying normal human tissue physiology and pathophysiology, and as new models for toxicity testing in the pharmaceutical and chemical industries. There is increasing scientific, ethical and economical pressure to use non-animal research methods and nowhere is this more apparent than in the consumer products industry where new legislation is forcing the development and adoption of non-animal research tools. This review focuses on how this industry is developing and adopting novel tissue engineering approaches that rise to the challenge of assuring consumer safety without animals.

Assuring the Safety of Consumer Products

Humans are exposed to a variety of chemicals in their everyday lives through their interaction with the environment and through the use of personal care, home care, food, pesticide and pharmaceutical products. It is a basic requirement that products used by consumers are safe under normal and reasonably foreseeable conditions of use. A large amount of legislation exists globally regarding the assessment of human safety for different product types (e.g., pharmaceuticals, pesticides, cosmetics, etc.). Whilst the details of this legislation can differ between countries and between industry sectors, the underlying principles of toxicological risk assessment prior to human exposure to a chemical are common. An assessment of the potential risk to human health is performed for each product prior to marketing, taking into account the general toxicological profile of the ingredients present within the product, their chemical structure, the level of human exposure and the target group who will use the product.

For each of the toxicological endpoints listed in Table 1, the risk assessment will comprise a number of pieces of information. The

*Correspondence to: Anthony M. Holmes; National Centre for the Replacement, Refinement and Reduction of Animals in Research; 20 Park Crescent; London W1B 1AL UK; Tel.: 44.0.20.7670.5331; Fax: 44.0.20.7670.5178; Email: anthony.holmes@nc3rs.org.uk

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risk to human health is generally regarded as a function of both the hazard represented by the chemicals present in the product and the amount of product to which the user is likely to be exposed. When performing a risk assessment for a consumer product the process involves four distinct phases.

Exposure assessment. An assessment of the amount and frequency of human exposure to the chemical under normal and foreseeable misuse conditions.

Hazard identification. The intrinsic properties of the chemical under consideration. Does the chemical have the potential to damage human health?

Hazard characterization. The relationship between the toxic response and the levels of exposure to the chemical. In many cases, a No Observed Adverse Effect Level (NOAEL) is determined i.e., the highest tested dose of a chemical that does not cause toxicity.

Risk characterization. The assessment of the risk that the chemical in question in the proposed use scenario will have adverse effects on human health.

For hazard identification and characterization, toxicological information will be required to provide data for risk assessments. In some cases, where hazard data for a specific toxicological endpoint are unavailable and these data are required to perform an adequate risk assessment, new data will be generated on the chemical. For each of the methods used to generate toxicological hazard identification and characterization data, OECD (Organization for Economic Co-operation and Development) guidelines are published.[3] The currently accepted methods for safety evaluation studies on chemicals within the European Union are published in the Test Methods Regulation.[4] Toxicology data derived from studies using animals can form a large part of the datasets used in the hazard identification and characterization for the safe use of chemicals. The UK's 2007 Statistics of Scientific Procedures on Living Animals gives the actual figure for the number of animals used in the 'protection of man, animals and the environment' across all sectors of industry at just over 150,000; 5% of the total number of animals used in licensed procedures in 2007.[5]

In the UK, the use of animals in research and testing is regulated by the Animals (Scientific Procedures) Act 1986. This states that wherever possible, testing on animals should be minimized, and this includes for the purpose of generating hazard identification and characterization data on chemicals. The use of alternative methods based on the 3Rs principles (Replacement, Reduction, Refinement; see Table 2) for the generation of toxicology data has a long history and has had some impact on animal use (see below). Unfortunately, this has not been a proactive process across all governments and industries and there have traditionally been few drivers to encourage the development and adoption of non-animal alternatives. Recently however, new European legislation, including the 7th Amendment to the Cosmetics Directive[6] and REACH (Registration, Evaluation, Authorization and Restriction of Chemicals)[7] have made it clear that a new approach is needed to reduce (and ultimately replace) animal tests in safety evaluation.

The safety of personal care products such as soaps, toothpastes, shower gels, mouthwashes, deodorants, shaving products, baby care products, perfumes and hair care products is covered by the EU Cosmetics Directive (76/768/EEC).[8] The 7th Amendment to this directive was published in March 2003[6] and sets out a number of points relating to the use of animals in the safety testing of personal care products and ingredients. Included in this, effective from September 2004, was an immediate ban on animal testing for finished personal care products. This is because it is recognized that the safety of finished products can be assessed based upon the hazard/risk characterization of ingredients that they contain and by methods that do not involve the use of animals. From March 2009, all animal testing of cosmetics ingredients within the EU was banned, and a marketing ban has been imposed on personal care products that contain ingredients that have been tested on animals after this date. In the case of three particular classes of animal toxicity tests (repeated-dose toxicity, reproductive toxicity and toxicokinetics tests) however, implementation of the marketing ban is delayed to March 2013.

Table 1 Toxicological endpoints (human safety) generally used in safety assessment[1]

- Acute toxicity
- Skin irritation and corrosivity
- Eye irritation and corrosivity
- Skin sensitization
- Dermal/percutaneous absorption
- Mutagenicity/genotoxicity
- Carcinogenicity
- Repeated dose toxicity
- Reproductive toxicity
- Toxicokinetics and metabolism
- Photo-induced toxicity

Table 2 The 3Rs

Replacement—methods which avoid or replace the use of animals in research that has the potential to cause them harm.

Reduction—methods which minimize animal use and enable researchers to obtain comparable levels of information from fewer animals or to obtain more information from the same number of animals.

Refinement—improvements to husbandry and procedures which minimize pain, suffering, distress or lasting harm and/or improve animal welfare in situations where the use of animals is unavoidable.

Current Status of Alternative Tests

As mentioned previously, the majority of accepted methods for generation of toxicological data within the EU currently rely on the use of animals. The development, validation and regulatory acceptance of alternative methods that can be used to replace, reduce or refine the use of animals in testing is an area which has received much attention. Within the European Union, the main role of ECVAM (European Centre for the Validation of Alternative Methods) is to coordinate the prevalidation, validation and independent assessment of alternative methods.[9] ICCVAM

(Interagency Coordination Committee on the Validation of Alternative Methods) and JaCVAM (Japanese Centre for the Validation of Alternative Methods) play similar roles in the validation of non-animal alternative tests in the USA and Japan respectively.[10] In April, 2009, a cooperation agreement was signed by ECVAM, ICCVAM, JaCVAM and Canada's Environmental Health Science and Research Bureau establishing an International Cooperation on Alternative Test Methods (ICATM) to enhance international cooperation and coordination on the scientific validation and evaluation of in-vitro toxicity testing methods. To date, several non-animal approaches have achieved validated status within the EU. These include tests for assessment of skin irritation, skin corrosion, phototoxicity, skin penetration and detection of severe eye irritants.

Skin corrosion and irritation. Validated in vitro tests exist for the assessment of skin corrosion and skin irritation that replace the Draize rabbit skin tests[11] developed in the 1940s. Many questions have been leveled at the Draize tests concerning the predictive ability and reliability of the model with respect to human irritancy and it has been shown that rabbit skin can both under-estimate for some chemicals and over-estimate for others the irritant potential in human skin.[12] The validated in vitro tests for skin corrosion and skin irritation employ three-dimensional models of human skin and therefore use tissue from the species most relevant for subsequent risk assessments.[13] These tissue engineered 3D models of human skin are commercially available (e.g., EPISKIN™,[14] SkinEthic RTE,[15] EpiDerm™[16]) and consist of normal, human-derived epidermal keratinocytes that have been cultured to form a multilayered, highly differentiated model of the human epidermis. These cells, which are cultured on specially prepared cell culture inserts using serum free medium, attain levels of differentiation and ultrastructure that closely parallel human skin. To test chemicals in these assays the material is topically applied to the engineered epidermis (as either a solid or a liquid) and cell viability is subsequently assessed using the MTT (a tetrazolium salt) assay.[17] In some protocols, the measurement of the inflammatory mediator interleukin-1α is included in addition to the measurement of cell viability. An OECD Test Guideline (TG) exists for the use of these 3D skin models for skin corrosion testing (OECD TG 431)[18] and ECVAM statements have recently been published regarding the validation of these models for skin irritation testing.[19] Discussions are currently ongoing regarding an OECD Test Guideline for the use of 3D skin models for skin irritation testing.[20]

Phototoxicity. If the skin is exposed to chemicals that absorb energy from sunlight, it must be ensured that this chemical excitation does not lead to adverse skin reactions. Many types of chemicals induce phototoxic effects and their common feature is the ability to absorb light energy within the sunlight spectrum. Therefore, before any biological testing is considered, a UV/visible absorption spectrum of the test chemical must be determined to indicate the wavelengths at which a compound may be susceptible to photochemical degradation. If a chemical is considered to have no photoreactive potential then no further tests are required. If however, a further test is needed, the neutral red uptake phototoxicity test using 3T3 Mouse Fibroblasts (OECD TG 432)[21] is a validated in vitro test that replaces a plethora of animal models previously used to assess phototoxicity (see Spielmann et al.[22] for review). To evaluate the phototoxic hazard in humans, a phototoxicity assay using a three-dimensional tissue engineered human skin model could be considered. These three-dimensional models allow concentrations of chemical more relevant to in vivo use to be applied directly to the culture surface and levels of UV light more representative of sunlight to be used.[23] In the past, these methods have been criticized for being expensive and time consuming, and their use has therefore been limited. Now however, three-dimensional skin models in 96-well plates have been developed for high-throughput screening making these a more economical and efficient option.

Skin penetration. Whilst an OECD guideline exists (OECD TG 427)[24] for in vivo animal testing to determine skin penetration, as mentioned earlier, the use of skin penetration data derived from in vitro systems is widely used in risk assessments for cosmetic products[25] and also has regulatory acceptance. The OECD Test Guideline (OECD TG 428)[26] recommends the use of excised human or porcine skin for the in vitro investigation of dermal absorption. Although the use of ex vivo porcine tissues for routine testing is not a regulated procedure it does still require animals and extrapolation of data derived from studies using these models; and a reliable source of human tissue for routine testing is not always available. The three-dimensional models of human skin mentioned above have been examined as a potential alternative source of tissue for use in skin penetration studies.[27-31] Such reconstructed skin models could be acceptable if their suitability can be shown by appropriate correlation studies. Unfortunately, at present, the barrier function of such models is not sufficiently well developed for use in penetration assays.[28,31] However, a number of strategies for improving the barrier properties of these human skin equivalents are being developed, including full-thickness tissues incorporating dermis and epidermis.[27,29,30]

Eye irritation. A large number of in vitro models exist for studying eye irritation, representing a substantial research effort to replace the Draize rabbit eye test. These assays can be grouped into ex vivo target organ/tissue assays [e.g., Bovine Corneal Opacity and Permeability Test (BCOP), Isolated Rabbit Enucleated Eye Test (REET, IRE), Isolated Chicken Eye Test (ICE)], organotypic models [e.g., the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) assay], cytotoxicity assays (e.g., Neutral Red Assays, Red Blood Cell Lysis Assay) and chemical reaction assays (e.g., EYTEX).[32] To date, none of these methods has met all the formal validation requirements for replacing the currently accepted Draize test in a regulatory context. However, both ICCVAM and ECVAM have recently stated that there is sufficient data to support the use of the BCOP and ICE (which use tissues obtained from abattoirs) in appropriate circumstances, and with certain limitations as screening tests to identify substances as ocular corrosives and severe irritants in a tiered testing strategy as part of a weight-of-evidence approach. Draft OECD Test Guidelines have been prepared for both assays and it is expected that formal adoption of the guidelines will occur in 2009. It is anticipated that no single in vitro assay will ultimately replace the use of animals in

the risk assessment process for eye irritation, but that a variety of non-animal models will be used. At present, there is considerable interest in the use of three-dimensional reconstructed tissue models (e.g., EpiOcular™[33] from MatTek and the SkinEthic[34] equivalent) of human cornea for the study of eye irritation.

Alternative Tests: Beyond Local Effects

Whilst the examples above show that use of non-animal methodologies using tissue engineering can be successful in avoiding the need for animals in the generation of toxicological data, these toxicological endpoints are primarily associated with local skin/eye effects. In the future it is plausible that new paradigms could be developed to enable risk assessment to support decisions on consumer safety of cosmetic and personal care products without the need to generate new data in animals.[35] Such new paradigms are unlikely to rely on direct one-for-one 'replacement' of the current animal studies used to provide data to make risk assessment decisions. The availability of technologies that did not exist ten years ago makes this new approach possible. Such techniques include both improved in vitro models (e.g., recent advances in tissue engineering primarily within the field of regenerative medicine) and technologies that allow far greater insights into changes within biological systems at a molecular level (e.g., transcriptomics, proteomics and metabonomics). Indeed, a recent report by the US National Research Council stated that: 'Advances in toxicogenomics, bioinformatics, systems biology, epigenetics and computational toxicology could transform toxicity testing from a system based on whole-animal testing to one founded primarily on in vitro methods that evaluate changes in biologic processes using cells, cell lines or cellular components, preferably of human origin.'[36]

A key component of such a new paradigm for risk assessment without the use of animals is the need for new models in which to characterize the adverse effects of chemicals. Already, as discussed in section two, the use of three-dimensional cultures of human skin (and to some extent corneal epithelium) has provided some very valuable approaches for the assessment of topical toxicity. The development of commercial human skin equivalents and the use of these and other three-dimensional skin models in toxicity testing has been possible because of the fundamental research to develop clinical products to treat burns victims. The breadth of research in tissue engineering means that the non-therapeutic application of tissue engineered models should not be limited to simple tissues such as skin and the cornea. Rapid advances are being made in three-dimensional engineering of more complex organs such as the liver and airways that could be integrated into a testing strategy. These models may not exactly mimic the in vivo situation for all aspects of an organ, but do they need to? Is it enough that these models are fit for purpose? For example, as mentioned above, the barrier function of three-dimensional human skin models is not yet optimal for studying skin penetration, but these same models provide valuable information on skin corrosion and skin irritation. Tissue-engineered models should be shown to be functionally relevant/equivalent to the biological process that is being studied.

Other advances that have allowed these complex culture models to be used in toxicological testing have been the standardization of the models and their commercial availability. These have facilitated their use in toxicology testing facilities that have expertise in studying the adverse effects of chemicals in vitro, but not in the production of tissue engineered culture models. This has enabled intra- and inter-laboratory variation of entire toxicology protocols to be evaluated in GLP-compliant trials using complex tissue models.

Can tissue engineered models help to define ways forward for risk assessment for more complex toxicological endpoints such as systemic or immunological effects? For example, animal tests for skin sensitization currently play a key role in the risk assessment process to ensure that exposure to chemicals does not induce skin allergy. Allergic contact dermatitis is a serious eczematous skin reaction resulting from a specific, delayed type hypersensitivity response to a small molecule (chemical) allergen.[37] Maxwell et al.[38] have recently published a proposal for a possible risk assessment framework for skin allergy that would not involve the use of animal tests. Such an approach aims to integrate data from several new models and technologies to study key aspects of consumer exposure (including the dermal kinetics of chemicals applied to the skin) together with information on effects on the underlying biology of skin sensitization such as chemical reactivity (e.g., in silico predictive models of chemical reactivity and chemical assessment of peptide reactivity) and immune cell activation (epidermal inflammation, dendritic cell activation and T-cell proliferation).

Characterizing the effects of chemicals on the adaptive immune response is one area where tissue engineering could play a role in overcoming the inherent limitations of traditional, static in vitro models. For example, in silico modelling of the induction of skin sensitization recently revealed the importance of trafficking of immune cells to the lymph node in regulating the sensitizer-specific proliferative response.[39] Immune cell trafficking is difficult to reproduce using traditional dendritic cell: T cell co-culture methods, however novel tissue engineering technologies such as the human artificial lymph node (ALN) described by Giese et al.[40] could provide more physiologically relevant in vitro approaches to characterize the effects and kinetics of chemicals on these pathways.

There are many other toxicological responses that are currently difficult (if not impossible) to study without the use of animals. In the area of systemic toxicity (i.e., the adverse effects that a chemical may have on the body if it enters the systemic circulation), many suggestions have been published regarding how this may ultimately be possible (reviewed in refs. 41 and 42), but as yet there are no examples of successful application of these approaches for risk assessment. The association between changes in target organs and systems, duration and frequency of exposure, administered dose and the absorption, distribution, metabolism and excretion (ADME) of the test compound cannot be considered in isolation as all of these parameters are inter-dependent. The development of alternative methods to determine the systemic toxicity of chemicals as well as to adequately predict their ADME properties, presents a considerable scientific and technical challenge. It is likely that an integrated approach to chronic toxicity testing based on the use of a range of alternative methods with complementary endpoints

would need to be developed. As well as information derived from laboratory studies using in vitro biological models, it is anticipated that risk assessments for such complex toxicological endpoints may also involve in silico modelling and informatics approaches to provide additional information and context that may be impossible to model in vitro.[33]

Building on the lessons that have been learned to date in the development of non-animal approaches for risk assessment, it appears that robust biological models that accurately represent key processes underlying human biology will be a major component in achieving success when developing novel risk assessment approaches for non-local effects. The toxic effects of a test chemical following systemic exposure can potentially be seen in any organ of the body or organ system. The question is can tissue engineered models based on human tissue be used to increase our understanding of the underlying biological processes behind some toxicological phenomena; and if so could this information be used to design more routine assays for use in testing chemicals. A further issue is how the tissue engineered product itself could be integrated into current tiered testing strategies for defining potential adverse effects of chemicals.

Conclusions

The authors believe the above discussions are reason enough to be optimistic that tissue engineering can play a significant part in assuring human safety without using animals. It is clear that significant challenges still exist and it would be naive to underestimate the difficulty in finding replacements for many toxicological endpoints. However it would be equally remiss not to explore and exploit the opportunities that are presented by tissue engineering to support scientific endeavor, protect human health and minimize the use of animals.

Some progress has already been made with three-dimensional models of human skin recently being accepted as alternatives for testing of skin irritation potential in the European Union. But can tissue engineering provide models for more complicated endpoints, such as systemic and immunotoxicity or toxicity of complex organ systems? Recent advances in bioreactor design may offer some hope. The development of more complicated bioreactors incorporating multiple chambers connected via a rudimentary vasculature is allowing scientists to conduct studies in vitro that would not otherwise have been possible due to the structure and function of such tissues being closer to that of the tissues in vivo than has previously been possible using two-dimensional cell culture. Human cells from different organs are cultured independently of each other in 3D, but connected via a constant flow of nutrient media which contains the test drug or compound, more closely mimicking the human body.[43,44] These bioreactors are enabling toxicologists to perform complex drug metabolism and absorption studies as well as more fundamental toxicity screening without using animals. Furthermore, developments in the shape of bioreactors are allowing researchers to control the size, shape and position of cells cultured as 3D structures, offering three-dimensional models with previously unattainable levels of complexity. Rago et al.[45,46] have devised a method that facilitates the recombination of cells grown independently of each other into micro-tissues that mimic the architecture of tissues/organs in the body. The increased functionality of these tissues make them ideal tools for toxicity testing and disease modelling which could reduce and eventually replace animal testing. Whether this is the case for all endpoints remains to be seen, but it is not unreasonable to think that future models based on these preliminary efforts will have the necessary functionality to do so. Incorporating these early models in current tiered testing strategies is essential to expedite this development and ensure confidence in this application of tissue engineering.

Translating the potential of tissue engineering into screening paradigms or using tissue engineering to overcome some of the limitations in animal models has particular resonance in the current climate of reducing costs and increasing efficiency. The challenge is to encourage clinically focused tissue engineers to consider this alternative application of the technology. Ensuring that communication channels between researchers developing these models and potential commercial end-users exist and remain open is essential for this to occur. Tissue engineering is moving forward very rapidly and it is often difficult for those not actively engaged to keep up with advances that could have implications for their own research. This is a problem that is becoming increasingly recognized with organizations such as the UK's National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) providing opportunities for tissue engineers from universities and small biotechnology companies to meet with their industry counterparts to explore potential collaborations and commercial uptake of tissue engineering. Identifying potential industry partners and the prospect of commercializing the technology is attractive, but is it enough to encourage researchers to modify their research focus? There need to be clear incentives to do this and there is no greater incentive than money. The NC3Rs has invested nearly half of its research funding budget (c. £4 million) in tissue engineering projects; and working with the Biotechnology and Biological Sciences Research Council (BBSRC) identified tissue engineering as a means to replace animals as a funding priority in 2007,[47] resulting in nearly £2.5 million being invested. Although a great deal of work is being done to recognize the potential of tissue engineering to bridge the gap between two-dimensional cell cultures and studies in animals/humans,[48] there is still a great deal more to do.

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