Inherent Risks Associated With Manufacture of Bioengineered Ocular Surface Tissue

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Objective: To review the potential health risks associated with bioengineered ocular surface tissue, which serves as a bellwether for other tissues.

Methods: All clinical trials using bioengineered ocular surface tissue published between July 1, 1996, and June 30, 2005, were reviewed with respect to materials used and statements of risk assessment, risk remediation, adverse events, manufacturing standards, and regulatory oversight.

Results: Ninety-five percent of investigational protocols used 1 or more animal-derived products and an overlapping 95% used 1 or more donor human tissues. Consideration of risks reveals a very low probability of potential harm but a significant risk of disability or death if such an event were to occur. Details of ethics approval, patient consent, and donor serologic test results were not consistently provided. No references were made to risk assessment or to codes of manufacturing and clinical practice.

Conclusion: While a degree of risk is associated with bioengineered ocular surface tissue, investigational reports of this new technology have yet to address issues of risk management and regulatory oversight.

Clinical Relevance: Attention to risk and codes of manufacturing and clinical practice will be required for advancement of the technology. We suggest the adoption of international standards to address these issues.

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The evolution of a routine, licensed protocol will require manufacturers to evaluate and remediate potential risks to patient safety. Thus, we have conducted a review of investigational protocols used in the preparation of bioengineered tissue for the ocular surface with the view to identifying potential risks along with measures for their remediation. In doing so, we highlight issues of relevance to emerging strategies for bioengineering other human cell products and draw attention to key guidance published in various jurisdictions.

METHODS

We reviewed all published accounts of human trials using ex vivo expanded epithelial stem cells (presumed), usually combined with, or applied to, a matrix carrier or substrate, for the reconstruction of the ocular surface during the period from July 1, 1996, to June 30, 2005. We reviewed the manufacturing procedures for the preparation of the bioengineered tissue and reviewed the known inherent risk of each documented step. This included the source and production of all components that had indirect or direct contact with each graft during preparation. We reviewed each publication for statements of ethical oversight, risk assessment, and techniques of risk remediation associated with these trials. Risk was defined as an event or consequence with a probability of occurrence leading to an impact on the health of the patient or wider society. In some cases, the probability of the occurrence of this risk was not known because it had not been measured or had not occurred but could not be considered impossible. We reviewed the availability of current regulatory documents with relevance to the preparation and clinical application of bioengineered tissue, where such information was available, from European, US, and Australian authorities.

RESULTS

Twenty human trials or case reports involving a total of 275 patients were published during the review period.10-29 These studies used ex vivo expanded presumed corneal, conjunctival, or oral mucosal epithelial stem cells for the management of ocular surface disease. Nineteen studies used various combinations of animal-derived products, including fetal bovine serum and other bovine-derived products, trypsin (source not documented but presumed to be porcine), cholera toxin, and feeder cell layers composed of irradiated or mitomycin C–treated murine 3T3 cells. Allogeneic human components, including limbal-corneal epithelial cells, human thrombin and fibrinogen, and amniotic membrane, were used in 19 studies. Details of donor serologic test results were provided in 4 publications. Autologous human products, including contralateral ocular surface cells, oral mucosa epithelium, and serum, were used in 17 studies (Table 1). The remaining materials are mainly standard research materials that are unlikely to have been labeled as "sterile and for therapeutic use" (a requirement under Good Manufacturing Practice), with the possible exception of insulin, antibiotics, and hydrocortisone, for which there exist pharmaceutical formulations but no details are given. The known and potential risks associated with materials used during graft preparation are listed in Table 2. Some risks have no documented probability but have occurred in similar settings (eg, cultured skin grafts). These risks should be assessed in context. The application of bioengineered skin in life-threatening conditions may warrant the acceptance of greater risk than sight-saving or cosmetic procedures.

Eleven studies used murine 3T3 cells to facilitate ex vivo expansion, but details regarding methods for growth inactivation are incomplete. As such, 4 studies stated use of gamma irradiation, but no details of dosage are given. Current practice for treating 3T3 fibroblast layers for cultured skin grafts uses 60 Gy of gamma radiation, although application is not standardized.34 Five studies stated use of mitomycin C at doses ranging from 4 µg/mL to 4 mg/mL for 2 hours, but the upper dose is suspected to be a typographical error at the time of publication (ie, should also be micrograms per milliliter). Cells treated by either irradiation or mitomycin C are described as "lethally irradiated" or "growth arrested." Three studies document the source of the 3T3 cells, but there was no evidence of in vitro testing of 3T3 feeder cells for contamination by murine viruses, bacteria, or tumorigenic potential prior to use.

Risk remediation has not been specifically addressed but there is some evidence of underlying concerns. For example, 1 protocol mentions the sourcing of fetal bovine serum from Australian and New Zealand herds, a practice that is consistent with guidelines issued by national regulatory agencies for managing the risk of TSE from bovine products (Table 3). Other bovine products used, such as pituitary extract, may also have been obtained from closed herds from geographically low-risk countries, although this is not documented.

Thirteen articles contained statements regarding ethics committee or institutional review board approval at a local level, but 16 make mention of acquiring patient consent. Six studies include a statement referring to the Declaration of Helsinki. No protocols made reference to national codes of Good Manufacturing Practice, Good Laboratory Practice, or current Good Clinical Practice or related guidelines. No international codes for manufacturing and clinical practice are known, but guidance documents are available on a limited number of specific issues (Table 3).

COMMENT

Published reports of patients treated with bioengineered ocular surface tissue began in 199720 and include work by 2 of us (I.R.S. and D.G.H.).11,13,24 Manufacturing standards reported in the investigational setting may be different from those required for licensing of protocols in major markets. We have reviewed the potential health risks to the individual patient and the broader society associated with this nascent technology. Such attention may also be relevant to many other tissues and organs in commercial manufacture as the processes of globalization and regenerative medicine progress and mature.

The known consequences of the murine 3T3 feeder layer include xenogenic microchimerism,3 xenotransplant rejection, and potential contamination with virus1 or prion agents26 during its production. There are potential risks...
from 3T3 feeder layers that have not been documented to date but remain present because of analogous risk from other fields such as xenotransplantation or embryonic stem cell investigation. Xenozoonosis,\textsuperscript{39,40} cell fusion,\textsuperscript{41} and tumorigenesis\textsuperscript{42} remain theoretical possibilities especially in light of the potential mutagenic effects of gamma irradiation and mitomycin C, which both affect DNA. There have been a number of historical precedents for animal-to-human disease transmission\textsuperscript{43-45} and this consequence may be more likely in patients receiving allogeneic grafts combined with immunosuppression. Furthermore, unrecognized and unknown viral agents may be present in the murine cells. A good example of such exposure was documented with the simian virus 40 contamination of poliovirus vaccine between 1955 and 1962. The virus was only recognized in 1960 and may have increased the risk of cancer in those who received the vaccine.\textsuperscript{30}

Any xenozoonosis is potentially lethal to the recipient and a greater unknown human community if the agent adapts to the human genome. Murine genomes encode endogenous retroviral sequences.\textsuperscript{46} Retroviruses are known

\textsuperscript{39}Wheeler T. The xenotransplantation paradox: identifying and controlling xenoviruses.\textsuperscript{40}The Xenotransplantation Paradox: Identifying and Controlling Xenoviruses, 2006.\textsuperscript{41}Kass EJ. The potential role of cell fusion in xenotransplantation.\textsuperscript{42}The Potential Role of Cell Fusion in Xenotransplantation, 2006.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
\textbf{Category} & \textbf{Component} & \textbf{No. (%) of Studies} \\
\hline
Autologous human products & Limbal epithelium & 11 (55) \\
& Conjunctival epithelium & 3 (15) \\
& Oral mucosa & 2 (10) \\
& Serum & 1 (5) \\
\hline
Allogeneic human products & Amniotic membrane & 16 (80) \\
& Limbal epithelium & 9 (45) \\
& Fibrin tissue adhesive & 1 (5) \\
\hline
Animal products (applied ex vivo) & Fetal bovine serum & 14 (70) \\
& Trypsin (suspected porcine but not specified) & 10 (50) \\
& Embryonic murine 3T3 cells & 11 (55) \\
& Growth arrested using mitomycin C & 5 (25) \\
& Growth arrested using gamma irradiation & 4 (20) \\
& Not specified & 2 (10) \\
& Bovine pituitary extract & 2 (10) \\
& Bovine aprotinin (component fibrin tissue adhesive) & 1 (5) \\
& Bovine insulin & 1 (5) \\
& Bovine collagen (collagen shield) & 1 (5) \\
\hline
Bacterial products (applied ex vivo) & Cholera toxin & 12 (60) \\
& Dispose & 7 (35) \\
& Thermolysin & 1 (5) \\
\hline
Antibiotics/antimycotics (applied ex vivo) & Penicillin & 8 (40) \\
& Streptomycin & 8 (40) \\
& Amphotericin B & 6 (30) \\
& Gentamycin & 4 (20) \\
& Neomycin & 2 (10) \\
& Ofloxacin & 1 (5) \\
\hline
Growth factors (applied ex vivo) & Epidermal growth factor & 10 (50) \\
& Insulin & 10 (50) \\
& Tri-iodothyronine (thyroid hormone) & 1 (5) \\
\hline
Culture media & Generic media & \\
& Dulbecco modified Eagle medium & 14 (70) \\
& Ham F12 medium & 11 (55) \\
& Specialist commercial media & \\
& Keratinocyte growth medium (BioWhittaker, Walkersville, Md) & 2 (10) \\
& Defined keratinocyte medium (Amniotech, San Francisco, Calif.) & 1 (5) \\
& Medium 165 (Kurabo, Osaka, Japan) & 1 (5) \\
& Medium 154 plus growth supplement (not specified) & 1 (5) \\
& Keratinocyte growth medium (Cascade Biologics, Inc, Portland, Ore) & 1 (5) \\
\hline
Miscellaneous & Hydrocortisone & 9 (45) \\
& EDTA & 9 (45) \\
& Phosphate-buffered saline & 7 (35) \\
& Dimethylsulfoxide & 6 (30) \\
& Glycerol & 3 (15) \\
& Glutamine & 3 (15) \\
& Transferrin & 3 (15) \\
& Nitrocellulose & 2 (10) \\
& Adenine & 2 (10) \\
& Selenium & 2 (10) \\
& Poly (N-isopropyl-acrylamide) & 1 (5) \\
& Petrolatum gauze & 1 (5) \\
& Ammonium salt & 1 (5) \\
& Hanks balanced salt solution & 1 (5) \\
\hline
\end{tabular}
\caption{Components Used in the Manufacture of Bioengineered Ocular Surface Tissue}
\end{table}
to integrate with host genomic DNA and transmit to all progeny. Such xenozoonosis has not been reported yet with keratinocyte ex vivo expansion, but such transmission could present devastating consequences to the individual recipient as well as the wider community.

On the basis of phylogenetic analysis, it has been shown that human endogenous retroviral sequences have a close relationship to the murine leukemia virus genus.47 Evidence exists that recombination due to exogenous infection of cells expressing endogenous retroviral sequences leads to generation of novel pathogenic strains in a feline model.48 Importantly, there are a number of murine viruses for which evidence of disease in man exists.49,50 This paradigm for concern is relevant given that more than half the protocols reviewed involved coculture of patient cells with murine 3T3 cells in the absence of normal immune defense functions. If this is seen as a remote risk, it is now believed that human immunodeficiency virus (HIV) is a xenozoonosis51 and perhaps even could be considered iatrogenic.52

Xenogenic microchimerism has been seen in at least half of patients receiving skin keratinocyte grafts,5 and these cells have been known to persist for at least 8 years.53 Because the 3T3 cell lines were originally established for the evaluation and testing of oncogenesis and because some 3T3 lines have lost contact inhibition,54 sourcing these cells becomes critical. It is thus encouraging that some groups have avoided use of 3T3 cells in the culture system. Nevertheless, the relative efficacy of cultures established in the presence or absence of 3T3 cells is unclear.

Current known methods of 3T3 growth inactivation (although not always documented) include irradiation by exposure to cobalt 60 or other gamma source. The radiation dosage used (60 Gy, based on skin culture protocols) is neither immediately lethal to cells55 nor antimicrobial.56 Furthermore, there may be little or no direct investigator knowledge of the cobalt 60 instrumentation and its calibration, process validation, dose mapping, or compensation for radioactive decay. Indeed, dose verification was not documented in any study. Treatment with mitomycin C has been used as an alternative to irradiation, although less is known about cellular lethality or antimicrobial effects of this treatment modality. Remediation of risks associated with the use of 3T3 cells could include tests for bacterial and viral contamination or tumor-forming capabilities. Human alternatives to murine 3T3 cells should also be considered, and mechanisms for encouraging cell expansion in the absence of any support cells should be emphasized as a research priority.

Bovine products have a variable probability of TSE infection that could be as high as 1.5 in 1 million.33 We suspect that most investigators used bovine products from closed herds residing in geographically low-risk coun-

<table>
<thead>
<tr>
<th>Consequence</th>
<th>Source (Caused By)</th>
<th>Event (Leading To)</th>
<th>Rate (If Known)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graft failure</td>
<td>Contamination</td>
<td>Inflammatory response</td>
<td>Varies 50% for skin grafts</td>
</tr>
<tr>
<td>Primary Secondary</td>
<td>Foreign antigen</td>
<td>Immune response</td>
<td>Varies 50% for skin grafts</td>
</tr>
<tr>
<td>Reduced vision</td>
<td>Local inflammation, infection, or tumor</td>
<td>Organ damage</td>
<td>Varies 50% for skin grafts</td>
</tr>
<tr>
<td>Reduced vision</td>
<td>Blindness</td>
<td>Systemic infection</td>
<td>Unknown</td>
</tr>
<tr>
<td>Loss of eye and/or disfigurement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
<td>Oncogene activation</td>
<td>Transformation of cultured cells</td>
<td>SV40 in poliovirus, rate controversial</td>
</tr>
<tr>
<td></td>
<td>Recombination</td>
<td>Feeder cells (eg, 3T3 cells)</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Growth factor related</td>
<td>Host tissue at graft site</td>
<td>Unknown</td>
</tr>
<tr>
<td>Human disease transmission (eg, HIV)</td>
<td>Contaminated material of donor human origin</td>
<td>Systemic infection</td>
<td>US tissue donors: HIV, 1 in 55 000; HCV, 1 in 34 000; HTLV, 1 in 128 000; corneal tissue: CJD, 1 in 1 million</td>
</tr>
<tr>
<td>Death</td>
<td>Foreign antigen contamination</td>
<td>Anaphylaxis</td>
<td>Bovine, 1 in 2 million</td>
</tr>
<tr>
<td>Human disease transmission</td>
<td>Contaminated material of animal origin</td>
<td>Systemic infection</td>
<td>TSE, 1.5 in 1 million</td>
</tr>
<tr>
<td>Xenozaonosis Known</td>
<td>Xenomicrochimerism as high as 50% for cultured skin grafts</td>
<td>Human disability and/or death</td>
<td>Unknown</td>
</tr>
<tr>
<td>Patient Community Emergent</td>
<td></td>
<td>Pandemic</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CJD, Creutzfeldt-Jakob disease; HCV, hepatitis C; HIV, human immunodeficiency virus; HTLV, human T-lymphotrophic virus; SV40, simian virus 40; TSE, transmissible spongiform encephalopathy.

*Consequences are in reference to ocular surface tissue. Rates refer to tissues where data are available (eg, skin).
tries (eg, Australia and New Zealand) thus reducing the risk further, but few details are available in published protocols. Additionally, some commercially available fibrin tissue adhesives produced from pooled donor plasma (being examined as a substrate for cell expansion and delivery) contain bovine aprotinin (reduces fibrinolysis), adding to the risk of microbial or prion contamination as mentioned earlier. Indeed, there have been reports of contamination with parvovirus. Moreover, purified bovine aprotinin itself has been documented as a source of allergy and even fatal anaphylaxis at a reported rate of perhaps 1 in 2 million. Broader research into the use of bovine products in research reagents reveals some un-

Table 3. Regulatory and Guidance Documents Relevant to Bioengineered Tissue Grafts*

<table>
<thead>
<tr>
<th>Organization</th>
<th>Notable or Informative Topics</th>
</tr>
</thead>
<tbody>
<tr>
<td>International Conference on Harmonization†</td>
<td>Identity and purity of cell banks; tests for karyology and tumorigenicity; Viral characterization</td>
</tr>
<tr>
<td>EMEA†</td>
<td>TSE guidance including tallow derivatives</td>
</tr>
<tr>
<td>Therapeutic Goods Administration (Australia)</td>
<td>Captures some cell-based products under the QSP for medicines</td>
</tr>
<tr>
<td>Code of Good Manufacturing Practice for Medicinal Products, Annex 2, 2002</td>
<td>TSE guidance and geographical risk for bovine spongiform encephalopathy</td>
</tr>
<tr>
<td>Code of Good Manufacturing Practice, Human Blood and Tissues, 2000</td>
<td>Donor selection and testing</td>
</tr>
<tr>
<td>Supplementary requirements for therapeutic goods for minimizing the risk of TSEs, 2004</td>
<td>General safety, sterility, and mycoplasma assays and other relevant issues</td>
</tr>
<tr>
<td>Food and Drug Administration (United States)</td>
<td>Source animal characterization</td>
</tr>
<tr>
<td>21 CFR §127034 (human tissue intended for transplantation)</td>
<td>Identity and purity of cell banks, tumorigenicity, and viral characterization</td>
</tr>
<tr>
<td>21 CFR §127134 (human cells, tissues, and cellular and tissue-based products)</td>
<td></td>
</tr>
<tr>
<td>21 CFR §6103 (general biological products standard)</td>
<td></td>
</tr>
<tr>
<td>Guidance for industry: Source animal, product, preclinical, and clinical issues concerning the use of xenotransplantation products in humans (CBER, 2003)</td>
<td></td>
</tr>
<tr>
<td>Points to consider in the characterization of cell lines used to produce biologicals (CBER, 1993)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CBER, Centre for Biologics Evaluation and Research; EMEA, European Medicines Evaluation Agency; QSP, quality system principles; TSE, transmissible spongiform encephalopathy.

*The guidance listed herein does not represent the complete thinking of each agency for these topics.
†May have been adopted by other jurisdictions, for example, Australia.

risks, although this is not known. Remediation for this product could include plant-derived trypsins and trypsin inhibitors. Cholera toxin, despite its name, was also assessed as low risk given the degree of purification, dose, and nature of use (culture medium supplement), but disease transmission remains theoretically possible, albeit unlikely. Isoproterenol, which mimics the actions of cholera toxin by also increasing the level of cyclic adenosine monophosphate in the cytoplasm, would therefore be preferable and has investigational support.

Contamination of donor human material with HIV, hepatitis C virus (HCV), human T-lymphotrophic virus, or other infectious agents remains rare, but risk is still inherent in these products. The estimated probability of viremia in tissue donors in the United States is reported to be 1 in 55,000 for HIV, 1 in 34,000 for HCV, and 1 in 128,000 for human T-lymphotrophic virus. The latency period for HIV immune response is up to at least 10 months even though the HIV viremia in those individuals is documented. At least 8 patients receiving various organs or tissues (outside of the presently reviewed studies) have been documented as having acquired HCV from a single donor who tested negative for this virus and was presumed to have been in the window between infection with HCV and development of a detectable HCV-antibody response. All allogeneic products would be subject to these risks of viral infections. Other pathogens, such as those responsible for TSEs, have been transmitted via purified pituitary hormone, corneal transplantation, and even blood transfusion and so must also be considered a potential inherent consequence of any allogeneic human tissue transplant. Remediation of risk
associated with human allogeneic products will require continued testing and vigilance.

No single guidance, regulation, or codified manual encompasses these issues. Even the Food and Drug Administration does not have a single document to provide standards or recommendations or a summary of requirements to investigators. Moreover, the final product often defies regulatory classification. All aspects and sourcing of such products beyond appropriate local institutional approval and informed consent from the individual patients should be considered on an international level. National and international regulatory agencies could provide more organized and definitive guidance because existing manufacturing guidelines are scattered and can be difficult to locate. An assembled list of key guidance documents is provided in Table 3.

To address the inconsistencies in ethics committee approval in these studies, we suggest an addendum to the Declaration of Helsinki to the effect that risk evaluation should be standard presentation in an application involving the transplantation of human and animal cell–derived products, including suitability of manufacture. In conclusion, investigational protocols for the manufacture of bioengineered ocular surface tissue have relied on the use of materials from animal and human donors, both of which carry varying levels of inherent risk to the individual and wider community. While no specific guidelines are available with reference to this technology, generic codes for manufacturing and clinical practice do exist on a national level along with associated national and international guidance documents. Published reports of clinical trials have neither made reference to these documents nor to formal methods of risk assessment and remediation. Nevertheless, it remains possible that such measures were taken in some if not all of the trials reviewed.

International guidance for scientists, surgeons, regulators, ethics committees, and even journal editors is essential to ensure that potential health risks are managed globally in a consistent and uniform manner. An effective approach could be through an addendum to the Declaration of Helsinki, but perhaps the best arena would be through the International Conference on Harmonization, which was borne out of a need to rationalize and integrate the global introduction of pharmaceuticals in response to health risks.

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REFERENCES
