Epidermolysis Bullosa: From Fundamental Molecular Biology to Clinical Therapies

Carol Featherstone[1]

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More than 70 international delegates participated in the "by invitation only" EB 2006 Conference,* organized by DebRA UK and DebRA Ireland and held in Dublin in October 2006. Since DebRA's last conference in 2000, exciting progress has been made in transforming a detailed knowledge of the fundamental molecular biology underlying the spectrum of genetic diseases known as epidermolysis bullosa (EB) into potential clinical applications. The conference aimed to review the current state of the art, to identify barriers to further progress, and to identify new avenues for future research, as well as to derive a consensus among the research and clinical communities on priorities for future research and development that will lead rapidly toward effective treatments. Researchers at the meeting reported the first case of successful gene therapy for EB as well as several approaches to improve gene therapies with new cellular delivery methods and improved gene therapy vectors, and the intriguing possibility of protein therapy for EB.

EB is a complex group of genetic disorders producing various degrees of skin blistering and shearing. It affects about one in 17,000 live births, with an estimated 500,000 cases worldwide. There are three main forms of the disease — EB simplex, junctional EB, and dystrophic EB — which are ascribed to mutations in ten well-characterized genes expressed at the dermal–epidermal junction (J Uitto and G Richard, *Clin Dermatol* 23:33–40, 2005).

Toward gene therapy

Michele De Luca (University of Modena, Italy, and Veneto Eye Bank Foundation, Venice, Italy) reported the first-ever successful gene therapy for EB. In a phase I/II clinical trial, he and his colleagues have "cured" areas of skin on the anterior thighs of a single, adult male patient with non-Herlitz junctional EB by transplanting several genetically modified epidermal sheets grown in culture onto both legs (a total of about 500 cm^2). The epidermal sheets were grown from the patient's own laminin 5-β3-chain-deficient epidermal stem cells taken from palm biopsies and transfected *ex vivo* with a retroviral vector expressing normal laminin 5-β3. The sheets were prepared by the same method as those already in use for the treatment of skin burns, and mucosal and corneal defects. The patient was a compound heterozygote expressing a low level of laminin 5 that is probably functional; he was selected for the trial because this basal level of protein production, the researchers believed, would protect against rejection of the graft. The grafts were applied to regions of the skin that were covered with several nonhealing lesions. The Italian surgical team prepared deep wound beds to receive the grafts by diathermy under local anesthetic. They saw complete epidermal regeneration of the patches on both legs after 8 days, and a normal-looking epidermis was maintained throughout the 1-year follow-up, from which they conclude that the stem cells were maintained in culture and renewed *in vivo*. They observed no MHC class I-restricted immune response and no antibodies against laminin 5. This work was reported recently in a paper in *Nature Medicine* (F Mavilio *et al.*, *Nat Med* **12**:1397–1402, 2007). Following on this "proof of principle," De Luca's team is planning the step-by-step replacement of a large proportion of this patient's skin surface with genetically modified skin; they also plan to coordinate a Europe-wide clinical trial with further patients.

Simply expressing the wild-type gene, as the Italian trial demonstrates, can work well for recessive mutations; however, the majority of the genetic defects in EB are dominant-negative mutations, meaning that a method must also be found to prevent expression of dominant mutants or to "dilute out" the dominant mutant with a large excess of normal protein. The teams of Irwin McLean (University of Dundee, UK) and Dennis Roop (Baylor College of Medicine, Houston, TX) have been working on such a strategy by using small interfering RNA to knock down expression of mutant keratin 14 in a mouse model of dominant EB simplex. They are trying three approaches: first, simply to knock down expression of the single mutant allele; second, to knock down both endogenous alleles and replace the mutant product by expressing a wild-type gene; and third, to switch on additional keratin genes to dilute out the mutant protein. They are currently developing vectors to express several short hairpin RNAs that are rapidly processed to small interfering RNAs that wipe out mutant keratin 14

[1]*Freelance science writer, Fourquevaux, France.*

Correspondence for DebRA Ireland: Dr Carol Featherstone, 19 Chemin d'En Peyroulié, 31450 Fourquevaux, France. E-mail: c.featherstone@wanadoo.fr. Correspondence for DebRA UK: Dr Clare Robinson, DebRA UK, DebRA House, 13 Wellington Business Park, Dukes Ride, Crowthorne, Berkshire RG45 6LS, United Kingdom. E-mail: clare.robinson@debra.org.uk.

gene expression. The same vector also expresses the normal keratin 14 gene, as well as a "fail-safe" mechanism to kill cells expressing the vector should anything go wrong. The problem of how to deliver such a construct to patients remains: an *ex vivo* approach like that of the genetically modified skin patches described above is being investigated in collaboration with De Luca, but it might also be possible to deliver it to skin cells *in vivo* using small-molecule vectors or viral vectors. Meanwhile, Roop has discovered, by using inducible mouse models for EB simplex and another keratin-based disease, epidermolytic hyperkeratosis, that simply increasing the ratio of wild-type to mutant keratin can ameliorate the disease phenotype. By coculturing epidermal stem cells expressing the wild-type protein with stem cells expressing the mutant protein, his team saw that cells expressing the wild-type protein have a selective advantage over cells expressing the mutant protein.

Improved gene therapies

Clinical trials for gene therapy have taken a setback since 2003, when two of the ten patients in a successful gene-therapy trial for severe combined immunodeficiency (M Cavazzana-Calvo *et al.*, *Science* 288:669–72, 2000) developed leukemia. This was the result of insertional mutagenesis caused by the retroviral vector integrating into the 5′ untranslated region of an oncogene. In light of this risk, the United States National Institutes of Health has produced recommendations for the design of ideal gene therapy vectors. Alain Hovnanian (University of Toulouse, France) reported the progress he and his collaborators in a large trans-European collaborative project have made toward developing a new generation of safe, self-inactivating (SIN) retroviral vectors modeled on these recommendations. These SIN vectors are based on lentiviruses, such as the human immunodeficiency virus, which tend to integrate into transcribed regions of genes rather than into the 5′ untranslated region. They contain SIN long terminal repeat sequences; a strong promoter specific for the gene they express, which can also impart tissue specificity to the transgene expression; and "insulator" elements that prevent transcription of neighboring DNA sequences and position effects such as silencing. Hovnanian's team has used such a vector to restore collagen VII expression and dermis–epidermis adherence *in vivo* to grafts of reconstructed skin. They are now looking at the tumorigenicity and immunogenicity of cells corrected with these vectors.

Another significant challenge in gene therapy for EB is the systemic delivery of the therapeutic gene — not only to the skin, but also to the cells lining the mouth, the rest of the gastrointestinal tract, and other affected internal sites. One potential solution to this problem may come from work described at the conference by David Woodley and Mei Chen (University of Southern California, Los Angeles, CA), who have simply injected genetically corrected autologous fibroblasts expressing wild-type collagen VII into the tail veins of recessive dystrophic EB mice with fresh skin wounds. From 1 to 8 weeks later, they saw the normal collagen VII protein produced by the corrected cells in the dermal-epidermal junction of the skin at the wound sites. The protein did not appear in unwounded skin, and when they expressed only the NC1 domain of collagen VII, it also did not appear at wound sites, which suggests some specificity. It is not clear whether the genetically modified fibroblasts home to the wound sites where they produce normal collagen VII or whether the protein itself accumulates at these sites from the blood; however, Woodley's and Chen's teams have also discovered that mouse or human collagen VII protein alone — purified from genetically corrected mouse fibroblasts — also appears in the dermal-epidermal junction of wounded skin after injection into the tail vein, where it appears to promote wound healing.

Although these remarkable observations generate further questions — the signal that attracts the genetically modified fibroblasts and the collagen VII protein needs to be identified, as does the mechanism of extravasation into the skin — these studies suggest that "protein therapy" and "cell therapy" approaches might be possible for EB as well as for ulcers and other skin wounds.

Woodley and Chen's findings might not be so surprising in light of the report by Katsuto Tamai (University of Osaka, Japan). He and his team found that, in mice, bone marrow stem cells expressing green fluorescent protein also find their way into the skin, where they presumably differentiate to produce new skin cells. This may explain how the skin has enough stem cells to sustain the healing process throughout the life of a severely affected EB patient. The green fluorescent protein-expressing bone marrow cells also appeared in skin grafts from collagen VII-null mice engrafted onto the green fluorescent protein-expressing mice, and Tamai estimates that up to 40% of cells in these grafted skin patches came from the bone marrow. Genetically corrected bone marrow stem cells may, one day, offer another route to whole-body gene therapy for EB.

Wound care

On the basis of the observation that fibroblasts from healthy donors generally do not provoke a strong immune response but they do produce collagen VII, Marcela Del Rio (Centro Comunitario de Sangre y Tejidos, Asturias, Spain) and her collaborators are developing a tissue-engineered "chimeric" skin equivalent, containing keratinocytes from dystrophic EB patients and healthy donor fibroblasts. They hope that the donor fibroblasts, although not immortal, will persist long enough, and will supply sufficient collagen VII, to make the skin-equivalent graft useful therapeutically without provoking rejection. Del Rio and her colleagues have a phase I/II clinical trial planned for 2007.

Paul Kemp (Intercytex, Cambridge, UK) described several products containing autologous or allogeneic fibroblasts that are currently in clinical trials to treat chronic wounds. He outlined problems in the processes of manufacturing, transporting, storing, and using living-cell therapeutics, for which Intercytex and other companies are developing solutions. He emphasized the need to design products with these practical considerations in mind: to determine the clinical strategy for their use early in the development process,

accepting the idea of a "gradual emergence of efficacy"; and to design the supply chain for the product during the clinical trials.

EB patients suffer chronic wounds, yet wound-management technology is still far from ideal. Edel O'Toole (Queen Mary's School of Medicine and Dentistry, London, UK) spoke about recent progress in developing skin substitutes, and "smart" systems such as nanoparticles and microspheres to deliver growth factors, drugs, and/or nucleic acids; as well as intensive work over the past 5 years on defensins (natural antimicrobial peptides) to treat infections. She was concerned that our knowledge of the role that factors such as stem cells, hypoxia, matrix metalloproteinases, and cytokines may play in EB wounds is still limited, and she indicated that wound healing in EB is a neglected research area.

Mark Ferguson (Renovo, Manchester, UK) described his team's elegant work on wound healing in fetuses *in utero* that led to the discovery that transforming growth factor-β3 (TGFβ3), which is high in fetal keratinocytes and fibroblasts but low in adults, dramatically improves the healing of surgical scars. By contrast, TGFβ1 and TGFβ2, secreted from degranulating platelets and inflammatory cells, are low in fetal wounds, but high in adult wounds. Pharmacological suppression of TGFβ1 and TGFβ2, or addition of exogenous TGFβ3 to adult surgical wounds, mimics scar-free fetal healing and results in much-improved surgical scars. All three TGFβ isoforms bind the same receptor but induce different conformation changes in the receptor that, presumably, induce different signaling cascades and attract different cell types to the wound. TGFβ3 plays a role in fibroblast migration; by using TGFβ3 to stimulate fibroblast migration into a healing wound, Ferguson and colleagues see a more normal "basket-weave" structuring of fibroblasts and secreted extracellular matrix proteins, and reduced scarring. Although there is no evidence yet that TGFβ3 might improve wound healing in EB patients, this work highlights the need for more research on growth factors and signaling cascades in EB wounds.

Drug discovery and development

Laurent Gagnoux (Institut National de la Santé et de la Recherche Médicale Unit 452, Faculty of Medicine, Nice, France) discussed matrix proteases and reminded the conference that, before the discovery of the genes affected in EB, these enzymes were the main focus of attention in EB research, and that they are implicated in the blistering process and tumor invasion. While analyzing the protein composition of junctional EB keratinocytes, he noticed that they lacked plasminogen activator inhibitor type 1 (PAI-1), which inhibits the conversion of plasminogen to plasmin by urokinase-type plasminogen activator (uPA). Plasmin, a serine protease, degrades many blood proteins, notably fibrin clots, but it also degrades the basement membrane, and overactivation of the uPA enzymatic cascade produces a junctional EB-like phenotype in transgenic mice. As the uPA inhibitor, PAI-1, appears to be absent from junctional EB keratinocytes, Gagnoux hypothesizes that inhibitors of uPA would help to control the symptoms of junctional EB. He is planning to test this hypothesis in cellular and mouse models of junctional EB, as well as to look at PAI-1 expression in keratinocytes from dystrophic EB patients.

In an attempt to identify drugs to treat EB simplex, McLean's team in Dundee, with the help of the Dundee Drug Discovery Unit, are screening small-molecule libraries for proteins that switch on gene expression from the keratin 6a gene promoter, with a view to switching on this normal keratin gene in EB simplex patients to substitute for the defective keratins 5 and 14. They have already identified seven molecules that turn down the keratin 6a gene promoter, which may be useful for treating other keratin-based diseases, but at the time of the conference they had not yet identified any upregulators. Academic researchers, McLean explained, may be willing to take on such "long-shot" drug discovery programs for rare diseases in which big pharmaceutical companies are unwilling to invest.

Marlene Haffner (Office of Orphan Products Development, United States Food and Drug Administration, Rockville, MD) expanded on this problem by describing moves in the United States and in Europe to facilitate the development of so-called "orphan" drugs and products. In the United States's Orphan Drug Act of 1983, an orphan product is defined as a drug or other product intended to treat a disease that affects fewer than 200,000 people (about seven people per 10,000) in the United States; in the European Union's equivalent act (2001), the definition applies to diseases that affect fewer than five people per 10,000 of the European Union population. Haffner described what criteria a product must meet to obtain orphan designation, which qualifies the sponsor of the product for tax credits and marketing incentives. She told the conference that about 1,500 products currently have orphan drug designation in the United States, of which 295 have received Food and Drug Administration approval to treat about 15 million patients with rare diseases in the United States. Among these, three products have been approved for EB.

There is a problem not only with commercial investment in drug development for rare diseases, but also in finding sufficient numbers of patients to participate in clinical trials. Here too, criteria for clinical trials of orphan drugs and products must be defined to assure their safety and efficacy. Brendan Buckley (European Centre for Clinical Trials in Rare Diseases, University College, Cork, Ireland) described these problems and spoke of the need for global registries of patients with rare diseases to help identify potential participants in transnational clinical trials.

Diagnosis

Such global registries presuppose that the patients registered in them have an accurate diagnosis of their disease. Giovanna Zambruno (Istituto Dermopatico dell'Immacolata, Rome, Italy) talked about the procedures and difficulties involved in obtaining an accurate molecular diagnosis of the many subtypes of EB. A molecular diagnosis generally begins with immunofluorescence and electron microscopy of tissues, followed by an analysis of the proteins and antigen mapping by immunofluorescence microscopy.

Subsequently, direct sequence analysis can detect almost 95% of mutations, but it is an expensive process, and it misses mutations in regulatory regions as well as large genomic rearrangements.

Birgit Lane (Centre for Molecular Medicine, Singapore) explained why the importance of accurate molecular diagnosis for the patient's benefit should not be underestimated: it fills the "information vacuum" for patients, helping them to come to terms with their disease, and it also helps in prognosis and improves genetic counseling for sufferers and carriers who wish to have children. Many of the mutation-based therapies that are being developed (the small interfering RNA approaches, for example) require knowledge of the specific mutations. Also, specific molecular diagnoses help researchers to understand disease mechanisms by developing animal and cell models, as well as looking at the biochemical properties of mutant proteins. Among EB simplex patients in the United Kingdom, she told the conference, about 20% have not had the underlying mutation identified.

Hiroshi Shimizu (Hokkaido University Graduate School of Medicine, Sapporo, Japan) confirmed that skin biopsies are still one of the most important steps for the correct diagnosis of the EB subtype. Biopsies allow pathologists to identify which layer of the skin the lesion occurs in, and they enable the protein deficiency to be identified with the use of monoclonal antibodies, thus allowing identification of candidate genes for further mutation analysis. Shimizu demonstrated how skin biopsies allow objective evaluation of the efficacy of treatment in his team's elegant studies of collagen XVII-null mice — the first, long-survived, transgenic model of junctional EB — in which the disease phenotype was cured by transfer of the human gene encoding collagen XVII.

Hiva Fassihi (St John's Institute of Dermatology, St Thomas' Hospital, London, UK) spoke about advances in prenatal diagnosis of EB for couples in which one or both partners are patients, or for couples who have already had one affected pregnancy. She described the possibilities and limitations of non-invasive prenatal diagnosis on fetal cells in the maternal circulation, and also of free fetal DNA in the plasma fraction of maternal blood, which represents 3%–6% of the total DNA in maternal plasma. The latter technique is already being used for X-linked disorders, to check the fetal rhesus D status and for some paternally transmitted single-gene disorders. Alternatively, cells removed from eight- to ten-cell-stage (day 3) embryos produced by *in vitro* fertilization have been used for preimplantation genetic diagnosis of a variety of conditions, including inherited skin fragility. Recently, preimplantation genetic diagnosis assays have been developed for severe forms of EB with the use of whole-genome amplification of DNA from single blastomeres followed by multiplex PCR of microsatellite markers close to the corresponding EB genes. This new approach is known as preimplantation genetic haplotyping. The United Kingdom's Human Fertilisation and Embryology Authority licensed specific preimplantation genetic haplotyping tests for the *LAMA3* and *LAMB3* genes in junctional EB in 2006.

Squamous-cell carcinoma

It remains unclear why dystrophic EB patients develop squamous-cell carcinoma (SCC) and why it is particularly aggressive. Ulrich Rodeck (Thomas Jefferson University, Philadelphia, PA) explained that the signaling pathways in sporadic SCC include EGFR, mitogen-activated protein kinases, phosphatidylinositol 3-kinase, and NF-κB, but whether these pathways also operate in the SCC seen in dystrophic EB is not known.

John Marshall (Barts and The London Queen Mary's School of Medicine and Dentistry, London, UK) is looking at the role of the epithelium-specific integrin $\alpha_v\beta_6$ in SCC. This integrin is normally expressed during development and generally not in normal adult epithelia, but it is also expressed *de novo* during wound healing and during tumorigenesis of colon cancers and breast cancers, among others. Marshall reported that $\alpha_v\beta_6$ is also upregulated in SCC from dystrophic EB patients and that it promotes tumor invasion in organ cultures. The mechanism by which $\alpha_v\beta_6$ promotes invasion may involve Cox-2, the enzyme that produces prostaglandins during inflammation, which is also upregulated in SCC. Marshall is looking at the effects of antibodies against the integrin's ligand-binding site as well as Cox-2 inhibitors on tumor growth *in vivo*.

Peter Marinkovich (Stanford University, Palo Alto, CA) is looking at the role of the basement membrane in SCC, specifically, the role of laminin 5, which is required for SCC development. Marinkovich and his team are currently investigating the structure–function relationships of laminin 5 domains — especially β3-chain and α3-chain domains — in cell migration and intracellular signaling. They find that a polyclonal antibody against the α3-chain G4–5 domain inhibits SCC growth in mice.

After the conference, an invited group of experts in dystrophic EB-linked SCC, chaired by Irene Leigh (University of Dundee, UK), met to brainstorm the main issues in fundamental and clinical research into this disease. This Skin Cancer Task Force identified the need for an international tissue bank and a more coordinated approach to testing potential chemoprophylactics. They stressed the need for a strategy for identifying the genetic and protein changes that arise at various stages of carcinogenesis in normal and dystrophic EB patients. They also noted the need for good consensus models for metastasis and invasion.

By bringing together many of the world's leading EB experts, this conference provided a valuable opportunity to exchange ideas on how best to move the field forward. Drawing on this impetus, DebRA intends to support the creation of further expert task forces addressing specific priorities for research and development. The next international DebRA conference, scheduled for 2009, should see reports of new clinical therapies developed from the promising progress reported in Dublin.

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