Molecular Basis of Epidermolysis Bullosa

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ABSTRACT

The dermal-epidermal anchoring complex is of critical importance in maintaining tissue integrity in the face of external shearing forces. Well-characterised important components include keratin 5 and 14, BP230, plectin, laminin 5, α6β4 integrin, BP180 and collagen VII. Epidermolysis bullosa simplex is due to mutation involving either keratin 5 or 14 or rarely plectin. Junctional epidermolysis bullosa is due to mutation involving laminin 5, α6β4 integrin or BP180. Dystrophic epidermolysis bullosa is due to mutation involving collagen VII. Understanding the molecular basis of the disease has a significant impact in diagnosis, counselling, prenatal and preimplantation testing, and opens up the possibility of gene therapy.

Keywords: *α6β4 integrin, BP180, BP230, collagen VII, dermal-epidermal junction, epidermolysis bullosa, keratin 5/14, laminin 5, plectin*

DERMAL-EPIDERMAL JUNCTION UNDER ELECTRON MICROSCOPY

Over the past decade, protein and genetic abnormalities have gradually been unveiled for most types of hereditary epidermolysis bullosa (EB). Much understanding has been gained regarding the underlying pathogenic mechanisms of this group of diseases. To appreciate these new advances and their clinical applications, it is important to understand the basic molecular organisation of the normal dermal-epidermal junction (DEJ). Figure 1 illustrates the structure of the DEJ under electron microscopy.

The central component of this complex is an electron-dense, intracellular, disc-shaped structure called the **hemidesmosome (HD)**. **Intermediate filaments (IF)** made up of keratin proteins utilise the HD as their major point of association with the dermal pole of basal epidermal cells. Proteins within the HD, including BP230 and plectin, link the cytoskeletal elements to transmembrane adhesion molecules. Fine filamentous structures, termed **anchoring filaments (AFil)**, project from the basal cell plasma membrane beneath the HD, traverse the lamina lucida and terminate within the lamina densa. The AFil appear to function in cell-matrix interactions involving laminin 5, the α6β4 integrin, BP180, and other molecules. Thicker cross-banded filamentous structures, the **anchoring fibrils (AF)**, originate in the lamina densa, extend into the subjacent connective tissue and terminate at structures known as anchoring plaques in the papillary dermis. The major structural component of the AF is type VII collagen. Figure 2 is a schematic model of the molecular organisation of the DEJ, illustrating the spatial relationships of the various molecules.

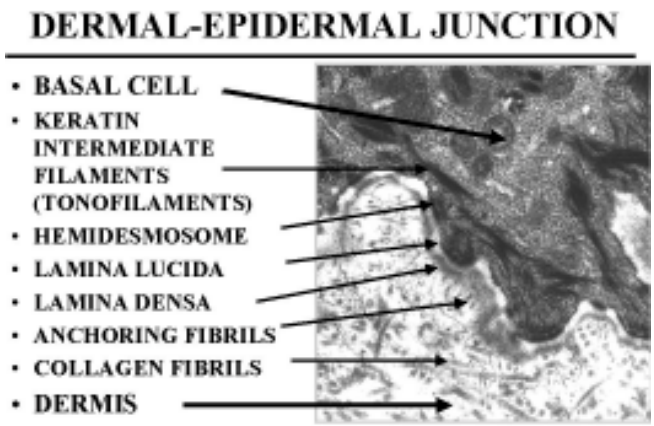

Figure 1: The DEJ under electron microscopy (Courtesy of Professor R.A.J. Eady, St John's Institute of Dermatology)

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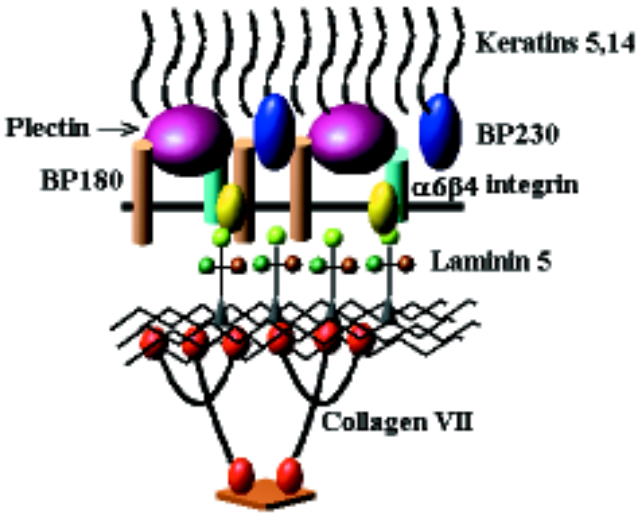

Figure 2: Schematic model of the molecular organisation of the dermal-epidermal basement membrane. The ability of α6β4 integrin and plectin to associate with each other and to self-polymerise facilitates the formation of a core that might serve as attachment site for BP180 and BP230. The stabilisation of the hemidesmosome is then achieved by multiple protein-protein interactions

MOLECULAR BASIS OF EPIDERMOLYSIS BULLOSA SIMPLEX

Keratins exist as obligate heterodimers of type I/II pairs,[1] for example keratin 5 pairs with keratin 14 in basal keratinocyte. Each keratin has its own specific partner(s).

Early studies by Anton-Lamprecht of skin from patients with Dowling-Meara type of epidermolysis bullosa simplex (EBS-DM) provided the first insight that EBS might be a keratin disorder. She noted that clumping of keratin filaments occurred very early in the pathogenic process, leading her to postulate that EBS might be due to a structural defect of the keratin.[2]

Subsequently various investigators discovered that truncated human keratin 14 (K14) protein disrupted the endogenous keratin network.[3] This pointed to the notion that keratin mutations may behave in a dominantly negative fashion to disrupt keratin filament assembly and architecture, in a way similar to what is seen in EBS-DM basal keratinocytes.

Vassar et al, using a reverse genetic approach, introduced one of the human mutant K14 genes into the germline of transgenic mice. In mice, the human keratin gene was appropriately expressed in the basal layer of the epidermis and in the oral epithelia. Mice expressing the mutant, but not the wild type human K14 gene, exhibited nearly all the phenotypic, morphological and biochemical traits characteristic of EBS-DM.[4]

Further studies were carried out in sequencing the corresponding genes from normal and EBS patients, and in conducting functional analyses of the defects. The majority of the EBS-DM cases analysed have mutations that reside in the α-helical rod domains of K5 and K14.[5,6] These regions play a critical role in the assembly of the K5/14 filaments. In contrast, mutations in EBS-Weber Cockayne tend to be found either in the head domain or in the L1-2 linker segment of K5, which are in less critical regions of keratin filament assembly.[6]

Taken together, these studies provided compelling evidence that: (1) structural defects in K14/5 genes can generate an EBS phenotype; and (2) the severity of the EBS phenotype correlated with the degree to which a specific K14/5 mutant perturbs the keratin filament assembly.

However, not all EBS patients have mutation in the keratin 5 and 14 genes. The plane of cleavage in EBS with late-onset muscular dystrophy (EBS-MD) is low intraepidermal, just above the plasma membrane in basal keratinocytes. Mutations in the gene encoding for plectin are found in this group of patients.[7] Most patients with this recessively inherited disease have associated premature termination codon-type mutations (PTC) and absent plectin expression.[8] The muscular dystrophy produced through ablation of plectin is understood since plectin is also immuno-localised to the sarcolemma of muscle cells, and may be involved in connecting the cytoskeleton to the sarcolemma. The reason for the late-onset of muscular dystrophy is however unexplained.

Table 1 summaries the mutational findings in the various major EBS subtypes identified to-date.

MOLECULAR BASIS OF JUNCTIONAL EPIDERMOLYSIS BULLOSA

Thus far, at least six genes have been implicated in the pathogenesis of the various forms of junctional epidermolysis bullosa (JEB).[9] The first mutation was reported only as recently as 1994. In a large number of

Table 1. Mutational analyses of inherited EBS

	EBS-WC	EBS-DM	EBS-MD
Usual mode of inheritance	AD	AD	AR
K5	mis	mis	N
K14	mis	mis	N
Plectin	N	N	PTC/PTC del/del

PTC premature termination codon
del deletion mutation
mis missense mutation
Adapted from Fuchs E (1999)[6]

patients with both the Herlitz and the non-Herlitz forms of JEB, specific mutations have been identified in the three genes encoding the constitutive polypeptides of the anchoring filament protein laminin 5, known as the α3, β3, and γ2 chains, and encoded by the *LAMA3, LAMB3,* and *LAMC2*, respectively. Mutations in the genes encoding other anchoring filament components have more recently been detected in other JEB subtypes. Mutations in the gene encoding both the α6 (ITA6) and β4 (ITB4) subunits of the epithelial cell specific α6β4 integrin have been detected in JEB patients who have co-existent pyloric atresia. Mutations in the gene encoding the BP180 (COL17A1) have also been demonstrated in some patients with JEB-nH.[10]

JEB, Herlitz type (JEB-H)

JEB is characterised by blistering at the level of the lamina lucida. Early electron microscopic data on JEB indicated ultrastructural abnormalities in the HD-AFil complexes, in which the hemidesmosomes were rudimentary and poorly formed.[11] In JEB-H, initial immunofluorescence staining for laminin 5 suggested the complete absence of this protein. Subsequent to the cloning of genes encoding the three polypeptides, mutation strategies have revealed genetic lesions in each of the three genes, *LAMA3, LAMB3,* and *LAMC2*. All mutations disclosed thus far result in premature termination codons, leading to complete absence of laminin 5 expression.[10] Laminin 5 is widely expressed in multiple tissue, and its absence in the Herlitz's variant results in respiratory, haematologic, genitourinary and gastrointestinal complications.

JEB, non-Herlitz type (JEB-nH)

Patients with JEB-nH have significant manifestations of junctional disease, but they do survive infancy and some improve clinically with age. They may have many of the cutaneous manifestations that are similar to the Herlitz variant, including periorificial erosion and significant nail/tooth/mucosal involvement, but are usually milder. Some of them appear to suffer from a reduction, but not a complete absence, of laminin 5 function. When analysed at the molecular level, this group of patients are shown to harbour a null type of mutation in one allele, and a structural mutation in the other allele, involving one of the three genes encoding for laminin 5.[12]

However, not all the JEB-nH patients have mutations involving laminin 5. Some of these patients analysed at the molecular level have been shown to have absent expression of BP180, resulting from underlying premature termination-type null mutations.[13,14] These patients belong to the group generalised atrophic benign epidermolysis bullosa (GABEB) in the previous classification but has been grouped in the JEB-nH subtype under the new revised classification.[15]

JEB with pyloric atresia (JEB-PA)

Immunofluorescence studies in the skin of patients with JEB-PA have revealed the absence or attenuation of staining with antibodies against α6β4 integrin. Mutations are demonstrated in the genes *ITGA6* and *ITGB4*.[16] A mutation in either integrin partner can give rise to the same JEB-PA phenotype. This disease usually involves PTC mutations, resulting in absent expression of functional α6β4 integrin heterodimers, although structural mutations have also been described.[10] The α6β4 integrin is present in a number of tissues, and its systemic absence in these patients usually results in death within the first few years of life. Some of these abnormalities include hydronephrosis and nephritis, and all these patients are born with pyloric atresia. Hemidesmosomes are severely hypoplastic in the skin of these patients, underscoring the critical role that α6β4 integrin plays in hemidesmosome assembly.

Table 2 summaries the mutational findings of the various major subtypes of JEB identified to-date.

MOLECULAR BASIS OF DYSTROPHIC EPIDERMOLYSIS BULLOSA

Under electron microscopy, dystrophic forms of EB (DEB) are characterised by the presence of

Table 2. Mutational analyses of various types of JEB

	JEB-H	JEB-nH	JEB-PA
Mode of inheritance	AR	AR	AR
Laminin-5 (LAMA3, LAMB3, LAMC2)	PTC/PTC	PTC/mis mis/mis del/del	N
BP180	N	PTC/PTC PTC/mis mis/mis del/del	N
α6 integrin chain	N	N	PTC/PTC
β4 integrin chain	N	N	PTC/PTC PTC/del PTC/mis mis/mis

PTC premature termination codon
del deletion mutation
mis missense mutation
Adapted from Pulkkinen L et al (1999)[10]

qualitative or quantitative abnormalities in anchoring fibrils.[17] Since anchoring fibrils consist predominantly, if not exclusively, of type VII collagen,[18] investigations were naturally steered along the line of *COL7A1* gene mutations as the cause in this group of patients. This was supported by immunohistochemical analysis of skin from patients with recessive dystrophic EB, which showed reduced or absent staining for type VII collagen epitopes along the dermal-epidermal junction.[19]

As to-date, all cases of DEB demonstrated at the molecular level are associated with mutations of the *COL7A1* gene.[20]

Recessive DEB, Hallopeau-Siemen type (RDEB-HS)

In the majority of the cases with this severe mutilating subtype RDEB-HS, the genetic lesions consist of PTC mutations in both alleles.[21] These mutations predict synthesis of truncated type VII collagen polypeptides, which are unable to assemble into functional anchoring fibrils. As a result, anchoring fibrils are absent, leading to severe fragility of the skin.

Recessive DEB, non Hallopeau-Siemen type (RDEB-nHS)

In the clinically milder subtype RDEB-nHS, the genetic lesion is frequently a missense mutation or in-frame deletion.[22] The mutation frequently affect a critical amino acid, which can alter the conformation of the protein and thus interfere with the assembly and packing of type VII collagen molecules into anchoring fibrils. Some ultrastructurally recognisable anchoring fibrils, although often morphologically altered, can still be detected.[17]

Dominant DEB (DDEB)

Type VII collagen is a homotrimer consisting of three identical chains. The characteristic genetic lesion in DDEB is a glycine substitution mutation in the collagenous domain of the type VII collagen, destabilising the triple helix molecule, exerting a dominant effect.[23] This interferes with the secretion and/or promotes intracellular degradation of the mutant molecules. Assuming equal level of expression from both the mutant and wild-type allele, one out of eight trimeric collagen molecules is expected to consist exclusively of normal polypeptides. The type VII collagen molecules in DDEB patients are apparently capable of forming anchoring fibrils, although they are usually thin and reduced in number due to faulty assembly of mutated molecules. Nonetheless, the presence of these structures explains the relatively mild clinical presentation in the dominantly inherited forms of DEB.

Table 3 summarises the mutational findings in the various forms of DEB.

Table 3. Mutational analyses of the major types of DEB

	DDEB	RDEB-HS	RDEB-nHS
Mode of inheritance	AD	AR	AR
Type VII collagen	GS del	PTC/PTC del/del mis/mis	GS/PTC mis/mis DTC/PTC GS/GS GS/del PTC/GS PTC/del del/mis

DTC delayed termination codon
GS glycine substitution
PTC premature termination codon
del deletion mutation
mis missense mutation
Adapted from Uitto J et al (1999)[20]

RELEVANCE OF MOLECULAR STUDIES TO BETTER PATIENT CARE

Ten years ago, essentially nothing was known of the underlying molecular mechanisms leading to blistering and other phenotypic manifestations of EB. In contrast, it is now well established that mutations in at least 10 distinct genes encoding the structural components of the DEJ underlie the different variants of EB (Table 4). The next question is whether the progress made in molecular aspects of EB will lead to better patient care. There are five areas in which molecular understanding of the diseases have made a significant impact in clinical care: diagnosis and prognosis, counselling, prenatal testing, preimplantation testing, and gene therapy.

Diagnosis and prognosis

Our understanding of the underlying mutations in different forms of EB provides guidance for refining the classification through identification of specific subgroups, with prognostic implications.

For example, identification of mutation in laminin 5 genes in a patient with JEB allows one to distinguish, on the molecular basis, whether the patient has a lethal, Herlitz type of JEB, or whether the disease is likely to be of the non-lethal variety. Similarly, in a patient with JEB-PA, detection of nonsense mutations in both *ITGB4* alleles will predict a severe disease, while the presence of missense mutations in the same gene will predict a relatively milder disease course.

Counselling

It is obviously pertinent to ascertain the risk of recurrence of the disease in future pregnancies in the same and in future generations. By current paradigm, the risk for recurrence in subsequent pregnancies of an autosomal recessive condition is 25%, while that of an autosomal dominant condition is 50%. But things are not always as straight-forward.

An example of the impact of molecular diagnostics is provided by observations on *COL7A1* mutations in the DEB. In particular, "sporadic" cases with relatively mild blistering tendency and clinically normal parents have long posed a diagnostic difficulty in genetic assessment and subsequent counselling. Specifically, such patients can be due to a relatively mild form of recessive dystrophic EB (RDEB), or they could equally be carrying a de novo dominant form of mutation. These two forms will be indistinguishable both clinically and by immunofluorescence and electron microscopic findings. With the ability to detect mutations in the specific gene, both in the patients and in the parents, the pattern of inheritance can be ascertained.

Another example is provided by Herlitz JEB, an autosomal recessive disease, which is characteristically a result of nonsense mutations in both alleles of any of the three laminin 5 genes. Thus the parents are expected to be obligate heterozygote carriers of the respective mutations, and the risk for recurrence in subsequent pregnancies of the same parents is 25%. However, in two recent reports of two such families, molecular examination of the mutations in laminin 5 genes in these

Table 4. Summary of the molecular heterogeneity of epidermolysis bullosa: clinical variants, level of tissue separation within the DEJ, and the mutated genes with corresponding chromosomal locations

EB variant	Level of tissue separation	Mutated gene	Chromosomal locus
EBS	Intraepidermal	KRT 5	12q11-13
		KRT 14	17q12-21
EBS-MD	Basal keratinocyte/Lamina lucida interface	PLN	8q24
JEB-PA		ITGA6	2q24-31
		ITGB4	17q25
JEB	Lamina lucida	COL17A1	10q24.3
		LAMA3	18q11.2
		LAMB3	1q32
		LAMC2	1q25-31
DEB	Sub-lamina densa	COL7A1	3p21.1

families have totally altered this risk estimation. Specifically, in both cases a newborn with severe Herlitz JEB with homozygous PTC mutations in both alleles of the *LAMB3* gene was noted. The mother in both cases was a heterozygote for the respective mutation, while the father did not carry the same mutation, yet non-paternity was excluded by microsatellite analysis. Careful haplotyping of the probands' DNA indicated that in both cases the disease had arisen on the basis of uniparental disomy, or inheritance of two copies of the gene from one parent. The most likely explanation of the genetic events leading to homozygosity of the mutation is meiotic non-disjunction followed by trisomy rescue. Whatever the real reason behind this genetic event, the important implications to the families are that these rare incidences are unlikely to happen in a subsequent pregnancy, and the risk for a similarly affected child is extremely small, much less than that of the predicted 25%.[24,25]

Prenatal testing

The risk of recurrence in subsequent pregnancies, be it the estimated 25% (for autosomal recessive conditions) or 50% (for autosomal dominant conditions), is still substantial. The ability to diagnose the condition prenatally is obviously important so that termination of pregnancy can be offered to the mother carrying a baby who has a severe condition like Herlitz JEB, Hallopeau-Siemens type of RDEB or JEB-PA.

In the 1980s, fetal skin biopsy played a major role in prenatal diagnosis to identify severe hereditary diseases.[26-28] The largest disadvantage of fetal skin biopsy is that they can be performed only during the second trimester of pregnancy at around 18-21 weeks of gestation, when the fetal skin is both morphologically and biochemically established. The parents with an at-risk fetus therefore have to face prolonged anxiety.

The elucidation of precise gene defects in families has led to the development of DNA-based prenatal diagnosis. A chorionic villus sampling at 10 weeks' gestation or amniocentesis at 13 weeks' gestation during the first trimester can be carried out to obtain fetal DNA for prenatal testing. The diagnosis can be made in 24-48 hours through DNA analysis, and the results obtained before the pregnant mother can actually feel the first fetal movement. Another advantage is the lower risk of such a procedure. The estimated fetal loss rate is 4-7% following fetal skin biopsy, 1% after chorionic villus sampling, and 0.5% after amniocentesis.[29] Needless to stress is that a first-trimester termination of an affected fetus is much less difficult and traumatic than a second-trimester termination.

Recent studies suggest that various fetal cells, such as trophoblasts, erythrocytes and leukocytes, circulate in maternal blood.[30] Among these fetal cells, nucleated erythrocytes appear to be suitable for prenatal diagnosis because they are uncommon in the peripheral blood of normal adults and are most abundant in the blood of the mother. Nucleated erythrocytes can be collected from the peripheral blood of pregnant women at 8-11 weeks' gestation. Contamination can be avoided with direct micromanipulation of single fetal nucleated erythrocytes under microscopic observation. Subsequently PCR amplification of these single cells and DNA analysis can then be carried out. This permits non-invasive prenatal genetic diagnoses. A difficult technical problem to overcome is to identify whether the selected cells are fetal or maternal in origin, especially when the fetus is female and does not carry a Y-chromosome.

Preimplantation diagnosis

Strictly speaking, prenatal diagnosis is not a form of disease prevention. Termination of pregnancy, when it is required, is performed because a "disease" has occurred. The couple still has to bare the psychological trauma of carrying but eventual loss of a potential baby.

Preimplantation diagnosis is an alternative to conventional prenatal diagnosis. It basically involves ovarian stimulation, ova retrieval and in vitro fertilisation. After successful fertilisation, a single blastomere biopsy is performed at the 6-10 cells stage of the embryo, ususally at about day three, and DNA analysis of the single blastomere is utilised for testing. Disease free embryos are selected for transfer to the uterus, thereby avoiding the need for termination of pregnancy.

From the various studies done so far, removal of one or two cells at this stage does not seem to affect the viability, cleavage rate or rate of development up to the blastocyte stage.[31] However it is premature to say that this procedure is definitely free from any long-lasting ill-effect to the potential baby.

The first successful application of a preimplantation genetic diagnosis was carried out for cystic fibrosis. Subsequently, this technique has been employed in a wide-range of hereditary disorders, such as Tay-Sachs disease, Marfan Syndrome, Hungtington chorea and familial adenomatous polyposis coli. Recently, trials have been carried out in families with Herlitz JEB.[32]

Preimplantation diagnosis, however, contains several disadvantages when compared to conventional DNA-based prenatal diagnosis. Technical difficulties, the higher cost and the low success rate for deliveries may make it less than attractive to some people. Nonetheless it is a valuable alternative to provide to couples who can afford the cost.

Gene therapy

The understanding of the genetic basis of various inherited diseases allows the insight of "cure" for these conditions by means of gene therapy.

To implement successful cutaneous gene therapy, several criteria must be fulfilled. Firstly, the corrective gene must be efficiently transferred to the cells of the skin, the basal keratinocytes in the case of EB. Secondly, stem cells must be effectively targeted during the gene transfer. Thirdly, adequate expression and precise localisation of the therapeutic gene product needs to take place. Finally, sustained expression of the corrective gene needs to occur over an extended period of time.

Theoretically, recessive type of EB can be corrected by replacing the patient's defective genes with a normal wide-type gene. For dominant condition, the mutant gene has to be "switched off" by knocking it off. However there is the distinct possibility of rejection of skin containing the therapeutic gene product, which might be recognised as a foreign protein by the patient's immune system. Immunosuppressive agents, topical or systemic, and ultraviolet light therapy, may be required as counter measures.

In theory, correcting the genes seems straight-forward. However, as to-date, successful gene therapy has eluded all the biomedical disciplines. Further research and trials are needed.

CONCLUSIONS

It has been more than a century since the first report of hereditary epidermolysis bullosa was described in the medical literature. Significant progress have been made, especially in the last two decades, of the molecular defects involved in the various major types of the disorder. However there are still many areas in which things are not totally clear. There are still some molecules, such as uncein and p300, the function and role of which are not clear. There are probably other proteins still unidentified. Knowledge of these may have significant impact on the success of future potential molecular treatment, and are not purely academic exercises.

The approach to classification of EB has changed in the light of molecular discovery of the underlying defect. Traditional classification based purely on clinical findings is unreliable and heavily observer-dependent. Variation in clinical manifestation within the same disease is not unexpected. Excessive subtypes based on clinical features alone may be made when in fact they may represent merely variation of the same disease process. This will lead to a lengthy classification system, which only serves to confuse, rather than help in the understanding and management of the disease.

However a classification based purely on molecular data, at least at present, is not totally satisfactory either. Similar or identical EB phenotypes may result from mutations involving different proteins, as in the case of non-Herlitz junctional EB. It is also clear that there may be little correlation between genotype and phenotype within at least some of the major types of EB. As such, a pure genetic approach will lead to a redundant and lengthy classification system, but may not add any important information. It is also obvious that molecular biological testing may not be available or affordable to some except in affluent countries with specialised centres. Strict reliance on molecular data for classification is plainly not feasible in many cases. Therefore, at present, it is best to adopt a combined clinical and molecular approach to classify EB.

The two major goals of management of an inherited disease are no different from those of any acquired disease: prevention when possible, and treatment when not. Understanding the underlying

mutations in EB has helped us to get closer to these goals. Immediately relevant to patient care are the improvements in accuracy of diagnosis, disease prognostication, and genetic counselling. The development of DNA-based prenatal diagnosis, which can be performed earlier and carries a lower fetal loss rate, is also a significant improvement over that provided by fetal skin biopsy. Preimplantation diagnosis through blastomere analysis, a technological advance that would obviate the necessity of termination of pregnancy in case of an affected fetus, represents the closest approach to the prevention of disease at present.

The future direction in the treatment of severe forms of epidermolysis bullosa is in gene therapy, by inserting and expressing new genetic information in somatic cells. The identification of mutations responsible for various phenotypes of EB has made it possible to consider gene therapy in this group of disorder. Despite the fact that keratinocytes offer an attractive target for therapeutic gene delivery, there are still many technical hurdles that have to be overcome. Improved methods for gene transfer, especially in vivo transfer, and achieving sustained high-level expression of the transgene are areas that need much research. As the field of epidermal gene therapy advances, hopefully one can look forward to a new dimension in the treatment of a wide variety of cutaneous disorders in the foreseeable future.

Learning points:

Understanding the underlying mutations in EB can improve the accuracy of diagnosis, disease prognosis, and genetic counselling. DNA-based prenatal diagnosis can be performed earlier and carries a lower tetal loss rate than fetal skin biopsy.

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