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Genetic Analysis of the Skin Disorder Weber-Cockayne Type Epidermolysis Simplex

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COLLEGE SCHOLARS PROJECT APPROVAL

Patrick Wade Clark
Scholar

Dr. Mary Ann Handel
Mentor

Genetic Analysis of the Skin Disorder Weber-Cockayne Type
Project Title and Completion Date
Epidermolysis Bullosa Simplex

COMMITTEE MEMBERS' SIGNATURES
(Minimum 3 Required)

Mary Ann Handel

Paul J Pantano

PLEASE ATTACH A COPY OF THE SENIOR PROJECT TO THIS SHEET AND RETURN BOTH TO THE PROGRAM DIRECTOR. THIS PAGE SHOULD BE DATED AND COMPLETED ON THE DATE THAT YOUR DEFENSE IS HELD.

DATE COMPLETED 5/5/03

Genetic Analysis of the Skin Disorder Weber-Cockayne Type Epidermolysis Bullosa Simplex

Patrick W. Clark
05/01/2003

Summary

Weber-Cockayne Type Epidermolysis Bullosa Simplex (EBS-WC) is a rare hereditary skin disorder characterized by intraepidermal blistering due to mechanical stress-induced cytolysis of the basal layer keratinocytes (8,14). In comparison to other autosomal dominant EBS subtypes, Weber-Cockayne is the least severe and is distinguished by localized blistering of the hands and feet (3). Previous research has linked EBS-WC to the basal layer keratin intermediate filament proteins, keratin 5 and keratin 14, and their corresponding genes KRT5 and KRT14, respectively (8,12). Due to the heterodimeric nature of the keratin intermediate filaments, mutations in KRT5 or KRT14 are responsible for the blistering phenotypes observed in EBS-WC patients (18). Linkage analysis of EBS-WC was performed by testing DNA samples from 16 members of an affected family. Oligonucleotide primers for keratin 14 and keratin 5 were chosen and polymerase chain reactions followed by gel electrophoresis of the amplified DNA fragments allowed polymorphic alleles to be analyzed. The banding patterns of the alleles indicated a strong linkage relationship between keratin 5 and affected individuals. In addition, linkage was not shown between keratin 14 and test subjects affected by EBS-WC. Based on this research, future DNA sequence analysis can be performed to identify the exact mutation in the KRT5 gene affecting this particular test family.

Introduction

Epidermolysis Bullosa (EB) encompasses a large group of hereditary skin disorders that are characterized by their level of split or separation in the skin layers (17). EB can be divided into three main types: EB simplex (with splitting within the basal layer keratinocytes), junctional EB (with separation within the lamina lucida) and dystrophic EB (with separation directly beneath the basement membrane, in the dermis) (17). EB simplex (EBS) is usually the most common form of EB and is divided into three different subtypes: Dowling Meara, Koebner, and Weber-Cockayne (1,2,3,7,16,19). Weber-Cockayne Type EBS (EBS-WC) is the focus of this research and is the mildest subtype characterized by localized blistering of the hands and feet (3,4,16). Both Dowling Meara and Koebner EBS are distinguished by generalized blistering of the entire body, and are often very debilitating (2,3,6,9,11,19).

The genetic basis of EBS-WC has evolved from several different procedures and techniques. Clumping and aberrations of the keratin intermediate filaments in basal epidermal keratinocytes typified early skin biopsies and ultrastructural analysis of EBS patients (11,13). The unusual clumping and disruption of keratin filaments preceded cell cytolysis and blister formation causing researchers to focus on the basal keratins (6,11).

One of the most important indications of basal layer keratinocytes being responsible for EBS was determined through reverse genetics experiments involving transgenic mice. Mice were introduced with either a KRT5 or a KRT14 mutation into their germ line and their subsequent phenotypes exhibited all the traits characteristic of EBS. In addition, transgenic mice studies were important in determining that not only the

location of the mutation within the keratin gene was important, but also the type of amino acid substitution was a critical factor in the severity of the disorder (2,4,20).

Linkage analysis is the method used in this research, and another important tool in determining the genetic basis of EBS-WC. In the three-generation test family, linkage analysis of the disorder in relation to polymorphic alleles within the mutated keratin gene allows EBS-WC to be traced through the pedigree. By linking the disorder to a particular allele, linkage to either KRT5 or KRT14 can be determined.

Unequivocal evidence that EBS-WC is caused by mutations in KRT5 or KRT14 was found by DNA sequencing of the corresponding genes from normal subjects and EBS patients (1). Previous research has identified a number of point mutations in either KRT5 or KRT14, which necessitates further research in the test family of this research.

Identification of keratin 5 or keratin 14 being the underlying cause of EBS-WC allowed tests with restriction fragment length polymorphisms (RFLP) to link EBS-WC to the keratin gene clusters on chromosome 12 and chromosome 17 (3). The KRT5 gene was determined to reside in the chromosome 12q11-q13 regions, and the KRT14 gene was isolated to the chromosome 17q12-q21 regions (15,18).

Subjects and Methods

Subjects

All participants gave their written informed consent to take part in this study (Appendix I). Buccal swabs were collected from 16 individuals in a three-generation family affected by EBS-WC and were prepared for DNA analysis. Out of the 16 individuals, 10 were affected by EBS-WC, and each subject was given a number designation to ensure confidentiality and unbiased analysis.

Buccal Cell Preps

To prepare buccal smear preps, 600uL of 50mM NaOH was added to each sample. The samples were vortexed and then heated at 95°C for 5 minutes. The brush was then removed from the sample and 60uL of 1M Tris (pH 8.0) was added to neutralize the sample. It was again vortexed and then spun in a centrifuge at 13,000 RPM for 5 minutes. The supernatant was removed and placed into a numbered tube for storage at -20°C.

Polymerase Chain Reaction (PCR)

PCR is a very sensitive test that enables strands of DNA to be amplified and visualized as distinct bands on various kinds of gels. One problem associated with PCR is no single protocol is appropriate for all situations. Therefore, for each set of oligonucleotide primers a new PCR application must be performed for optimal results. Some of the must common problems include: no detectable product, a low yield of the desired product, and nonspecific background bands and smears due to the formation of primer-dimers that are incorrectly amplified. The primers chosen for both keratin 14 and keratin 5 required different protocols and parameters that required repeated tests of trial and error to yield the best results.

The enzyme used in the PCR was *Taq* DNA polymerase. The enzyme concentration is important because if the concentration is too high, nonspecific background products may accumulate, and if too low, not enough desired product will be made (10). The four dNTPs were used in equal amounts at low concentrations to minimize mispriming at nontarget sites and to reduce the probability of creating altered DNA sequences. In addition, the concentration of magnesium had to be adjusted to

optimize desired product. The concentration of magnesium may affect the following: primer annealing, product specificity, formation of primer-dimers and enzyme activity and fidelity (10).

The temperature and length of time required for primer annealing was also altered based on several reasons. The optimum temperature for primers to anneal (Tm) was found by the following calculation:

Tm =Th -5°C

Th = [9 (C/G) x 4°C] + [11 (A/T) x 2°C]

An accurate annealing temperature is actually 5°C below the true Tm of the designated primers. Raising the annealing temperature of the PCR enhances discrimination against incorrectly annealed primers and decreases the extension of incorrect nucleotides at the 3' end of the primers. In contrast, low annealing temperatures and high dNTP concentrations favors misincorporation of incorrect nucleotides (10).

The concentration of primers was another variable that had to be adjusted for desired yields. If primer concentrations were too high, they promoted mispriming and the formation of nonspecific product and primer-dimers. These misincorporations are themselves substrates for PCR and compete with the desired product for enzyme, dNTPs, and primers, resulting in decreased yields of the desired product (10).

Two sets of primers from previously published studies specific for the KRT14 gene were chosen that had a high rate of polymorphisms in the population. A tetranucleotide repeat polymorphism at D17S846 was chosen that had sufficient variation for linkage analysis (5). The forward primer for KRT14 was designated 17-846-1 (5'-TGC ATA CCT GTA CTA CTT CAG-3') and the reverse primer was designated 17-

846-2 (5'-TCC TTT GTT GCA GAT TTC TTC-3'). The second set of primers was designated, forward primer 17-800-1 (5'- GGT CTC ATC CAT CAG GTT TT –3') and the reverse primer 17-800-2 (5'- ATA GAC TGT GTA CTG GGC ATT GA – 3') (OMIM 148066).

Allele	bp	Frequency		Allele	bp	Frequency
A1	255	0.01		A6	235	0.22
A2	251	0.04		A7	231	0.09
A3	247	0.06		A8	227	0.07
A4	243	0.27		A9	223	0.01
A5	239	0.22		A10	215	0.01

Figure1. Alleles and frequency of the tetranucleotide repeat polymorphisms amplified by the primers 17-846-1 and 17-846-2 (5).

Each PCR sample was run at the following parameters: 2.5 uL of 10X buffer, 7.75uL of water, 2uL of each primer, 1uL of each nucleotide, and 0.25uL of *Taq* DNA polymerase. The cycle was run at 94°C for 6 minutes (initial denaturation), 94°C for 1 minute, 50°C for 1 minute (annealing), 72°C for 1 minute (extension), held at 4°C and repeated for 30 cycles in a DNA thermal cycler.

Primer pairs for KRT5 were designed from the NCBI website (GenBank accession number NT009609) that included a single nucleotide polymorphism (SNP). The SNP, rs631988, has a cytosine to guanine (C/G) polymorphism. There were two forward primers representing the C/G polymorphism and were designated Ker5-2 (5'-TGA TTC CAC TTC TTC CCA G**C**-3') and Ker5-3 (5'- TGA TTC CAC TTC TTC CCA G**G**-3'). The reverse primer for the KRT5 gene was designated Ker5-1 (5' –TCA CAG GAG AGA AAA GAA CA-3'). Each PCR sample was run at the following parameters: 2.5uL 10X buffer, 7.75 uL of water, 1uL of Ker5-1 and 1uL of Ker5-2 or

Ker5-3 primers, 1uL of each nucleotide, and 0.25uL of *Taq* DNA polymerase. The cycle was run at 94°C for 6 minutes (initial denaturation), 94°C for 1 minute, 53°C for 1 minute (annealing), 72°C for 1 minute (extension), held at 4°C and repeated for 30 cycles in a DNA thermal cycler.

Gel Electrophoresis

DNA is chemically an acid (takes a negative charge in solution) due to its phosphate groups. Thus when an electrical field is applied to the DNA samples that have been amplified by PCR, they migrate toward the positive charge or anode. Migration distances are inversely proportional to the samples' molecular weight. 10uL of each PCR reaction sample and 2uL of loading dye were inserted into wells in the top of a 10% TBE gel and the samples were run at 100V for approximately 45 minutes in 1 X TBE solution. The gels were then stained in ethidium bromide for 5 minutes and viewed with ultraviolet light.

DNA Sequencing Gel

The high frequency of polymorphisms and the tetranucleotide repeat in the alleles specified by the KRT14 primers, 17-846-1 and 17-846-2, required a DNA sequencing gel to specify each allele. The 10% TBE gels did not allow for very specific banding patterns, where as the sequencing gels allowed for more specificity. A PCR reaction was performed according to the parameters specified above for KRT14 with the addition of 1uL of radioactive P32 to each sample to label the DNA. The sequencing gel was run and exposed for varying lengths of time to yield optimum banding patterns.

The DNA sequencing gel was not run for the KRT5 alleles because heterozygotes and homozygotes could be determined from the normal 10% TBE gels.

Results

To determine the gene responsible for EBS-WC in the test family, linkage analysis was performed for KRT14 and KRT5. Selecting primers that had sufficient variation in the population was critical to the success of the study. The first gene studied was KRT14 and two different sets of primer pairs specific for the KRT14 gene sequence were chosen. The first set of primers 17-800-1 and 17-800-2, for reasons specified in the above methods, had either no detectable product or very low yields. Due to the lack of results from primers 17-800-1 and 17-800-2, the next set of primers 17-846-1 and 17-846-2 were used at an annealing temperature of 53°C and a concentration of 2uL per test sample. Primers 17-846-1 and 17-846-2 yielded detectable amounts of products with distinct bands.

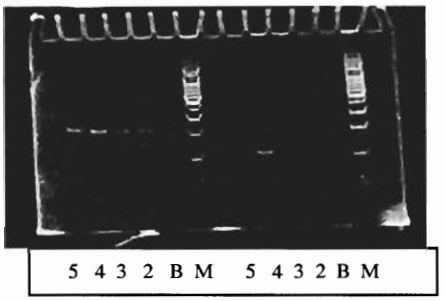

Figure 2. From right to left the gel picture shows primers 17-800 and then 17-846. The gel lanes are labeled according to the corresponding DNA sample number (M indicates the DNA band size marker and B indicates a blank). The gel shows the low yield associated with primers 17-800. Also, the bands are of length ~225 bp which is the expected band size.

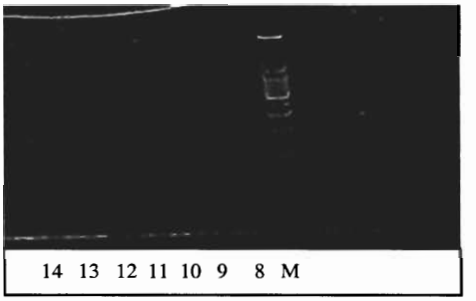

Figure 3. From right to left the gel picture shows primers 17-846. The lanes are labeled according to their corresponding DNA sample number (8-14).

The 10% TBE gels did not allow for determination of different alleles within the test family, so a DNA sequencing gel had to be performed. The sequencing gel identified 5 different alleles for the amplified region of KRT14. Each subject was labeled with their corresponding allele and pedigree analysis showed no linkage between EBS-WC and the KRT14 gene.

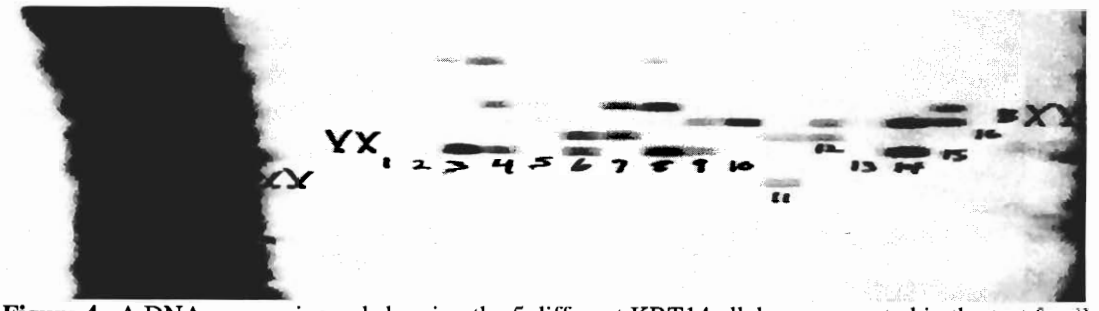

Figure 4. A DNA sequencing gel showing the 5 different KRT14 alleles represented in the test family. Through pedigree analysis KRT14 showed no linkage to EBS-WC.

Polymorphic Alleles in KRT14

Sample #	1	2	3	4	5
1 –N	---	----	----	----	----
2 –EBS		+			+
3 –N	----	----	----	----	----
4 -N		+			
5 –EBS		+			+
6 –EBS				+	+
7 –N		+	+		
8 –EBS			+		+
9 –EBS		+			+
10 –EBS		+		+	
11 –EBS			+	+	
12 –N	+		+		
13 –EBS			+	+	
14 –N	+			+	
15 –EBS		+		+	
16 -EBS				+	+

Figure 5. The test subjects' phenotype designations along with their number designations are shown in the sample # column. Analysis of allele segregation shows no linkage between EBS-WC and KRT14. To show linkage, a specific allele would have to correspond to all affected subjects ('+' indicates presence of allele in subject).

Since no linkage between KRT14 and EBS-WC was found in the test family, primers for the KRT5 gene were created that contained a single nucleotide polymorphism (SNP) that allowed linkage analysis to be performed.

The primers K5-1 and K5-2 amplified the cytosine residue and the corresponding allele was designated by the numeral 2. The primers K5-1 and K5-3 amplified the guanine residue and the corresponding allele was designated by the numeral 3.

The following electrophoresis gel pictures show the banding patterns of each subject, showing either heterozygousity or homozygousity for the SNP. For example,

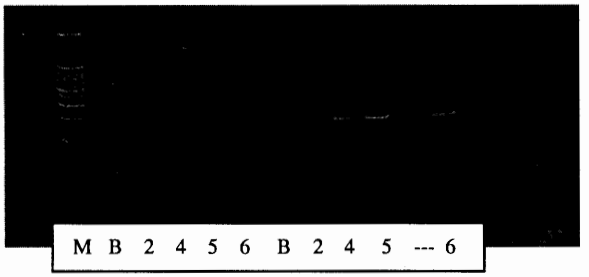

Figure 6. From left to right, the gel shows K5-1 and K5-2 primers, test samples 2,4-6, representing allele 2. Next, primers K5-1 and K5-3, test subjects 2,4-6, represent allele 3.

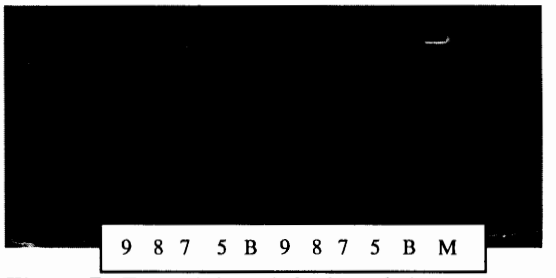

Figure 7. From right to left, the gel shows the maker then K5-1 and K5-2 primers, test samples B,5,7,8,9, representing allele 2. Next, primers K5-1 and K5-3, test samples B,5,7,8,9, representing allele 3.

the gel pictures show a band present for the 3 allele, but not the 2 allele for test subject 5. Therefore, test subject 5 is homozygous for the guanine residue allele number 3. Allele determination was performed for each test subject and the allele number was designated on a pedigree for the test family.

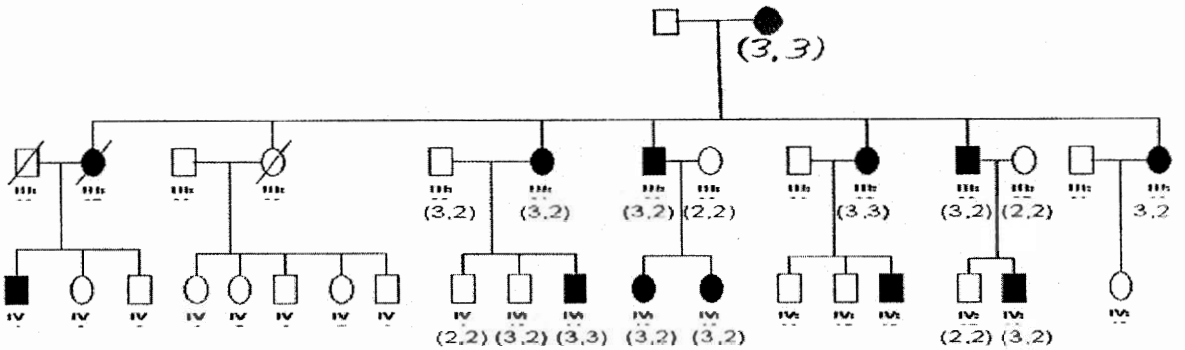

Figure 8. All 16-test subjects in the three-generation family were labeled with their subsequent allele designation. The pedigree shows that EBS-WC is linked to allele 3 in the test family. Some unaffected subjects are heterozygous containing allele 3, but inheritance is from an individual outside of the test family (squares represent males, circles represent females, and darkened symbols represent affected subjects).

The pedigree shows that all subjects affected with EBS-WC are either heterozygous [2,3] or homozygous [3,3], but all unaffected subjects are either heterozygous [2,3] or homozygous [2,2]. Therefore, inheritance of EBS-WC is shown to follow allele 3 thus, linking KRT5 to the disorder.

Discussion

The results indicate a strong probability that KRT5 is linked to EBS-WC in the test family. The allele designated by the numeral 3 shows linkage only within affected individuals. The heterozygous unaffected individuals also carry a 3 allele but inheritance of the allele is from individuals outside of the affected family. Family members that are related by marriage allow more alleles to be distributed to subsequent offspring and better linkage analysis results. The pedigree shows that the 3 allele from unaffected heterozygous subjects does not pass on EBS-WC. However, the pedigree does show a direct correlation between EBS-WC and inheritance of the 3 allele from affected individuals.

The KRT14 gene was not linked to EBS-WC by the 5 polymorphic alleles amplified by PCR. The three-generation pedigree allowed determination that no allele from affected individuals linked to EBS-WC. The fact that KRT5 is linked and KRT14 is not linked corresponds to previous studies that indicate only one keratin mutation for the majority of EBS-WC cases (3,8,9,18). Due to the heterodimeric nature of the keratin 5 and keratin 14 intermediate filaments, it is only plausible that one of the keratin genes would contain a disruptive mutation and not both.

Future DNA sequence analysis of the KRT5 gene in the test family will hopefully reveal the exact mutation responsible for EBS-WC. As more information is gained regarding EBS-WC, the more probable a cure or treatment will be discovered.

Acknowledgements

This research has been the culmination of a life-long series of events and struggles with EBS-WC. I would like to thank Dr. Mary Ann Handel, my faculty mentor,

for all of her guidance and support. She allowed the project to get started and supported my research wholeheartedly. The majority of this research took place at the University of Tennessee, Medical Center Genetics Laboratory under the guidance of Dr. Karla Matteson. I would like to thank Dr. Matteson for so generously giving of her time and her lab's resources to help with my research. Next, I would like to thank Dr. Paul Pinckney for serving on my project committee and always serving as a mentor and friend. Also, the support and cooperation of all of my family members that participated in the project is greatly appreciated. Finally, I would like to dedicate this project to my Grandmother, Mrs. Virginia Spraggins, who gratefully participated in the project, but in dept regret failed to see its completion.

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Appendix I

The Use of Cheek Cells as a Source of DNA in the Genetic Mapping of the Skin Disorder Weber-Cockayne Type Epidermolysis Bullosa Simplex

Introduction

This letter is to inform you about an opportunity to participate in a graduate level research study. The research is being performed as part of a graduate thesis. The study will try to determine the DNA mutation that is responsible for causing the skin disorder characterized by blistering of the hands and feet known as Weber-Cockayne Type Epidermolysis Bullosa Simplex in our family. If the study is successful, the chromosome location and the type of DNA mutation responsible for the disorder will be identified. However, there is no guarantee that the research study will reveal any DNA mutations. In addition, studies of Weber-Cockayne Type Epidermolysis Bullosa Simplex have been previously performed, and the DNA mutations have been identified in other families. Thus, this study is of interest to our family and will provide an opportunity for Patrick Clark to train in this type of research, but is unlikely to provide a major step forward in the knowledge of this disorder.

Information about your involvement in the study

If you choose to be involved in this study, the following will take place: first, read and fully understand this form, next, sign the form in the presence of a witness, have the witness sign the same form, and return it to Patrick Clark, lastly, a soft brush (swab) will be used to collect cells from the inside of your mouth on the cheek. The swab will be brushed on the inside of both cheeks for approximately 30 seconds. This procedure will be preformed twice in case of contamination of the DNA. The participant will perform the procedure and the swab will be returned to Patrick Clark. The DNA will be extracted form these cells in order to study the genetic skin disorder.

No additional procedures, no additional costs are necessary.

Risks

The collection of cheek cells on the swab is a routine non-invasive procedure, and we anticipate no measurable risk to you.

Maintaining confidentiality is of the highest importance and the appropriate steps will be taken to ensure your privacy. At no time will genetic findings be discussed, reported, or published that are not relevant to the skin disorder.

In this study, an inheritable gene will be studied which requires the testing of both parents and children. Thus, this testing could reveal paternity issues that are not public knowledge.

Confidentiality

The information in the study will be kept confidential. Data will be stored securely and will be made available only to persons conducting the study. No reference

will be made in oral or written reports that could link participants to the study. In addition, any DNA information obtained will not be added to any forensic databases.

Benefits

There is no direct or immediate benefit to you. A benefit of the study will be locating the gene that is responsible for causing the blistering of the hands and feet. However, previous studies have already located the DNA mutation for Weber-Cockayne Type Epidermolysis Bullosa.

Compensation

No compensation is available for voluntary participants of this study. Appreciation, however, will be immeasurable.

Contact information

If you have questions at any time about the study or the procedures (or you have experienced adverse effects as a result of participating in this study), you may contact the researcher: Dr. Karla J. Matteson at 865-544-9449. If you have any questions about your rights as a participant, contact the Compliance Section of the Office of Research at 865-974-3466.

Participation

Your participation in this study is voluntary; you may decline to participate without penalty or question. If you decide to participate, you may withdraw from the study at any time.

.....

Consent

I have read the above information. I have been informed and understand that this type of analysis can detect when a child's biological father is not the same as the current male parent (non-paternity). I have received a copy of this form. I agree to participate in this study.

Participant's Signature:_____ Date:_____

Parent's Signature (if under 18):_____ Date:_____

Witness's Signature:_____ Date:_____

Appendix II

The contents of this paper were submitted for credit in BCMB 409: Perspectives in BCMB on 11/19/2002.

Genetic and Molecular Analysis of the Skin, Emphasizing the Skin Disorder Epidermolysis Bullosa Simplex

Introduction

Epidermolysis bullosa simplex (EBS) is a group of rare genetic skin disorders characterized by intraepidermal cytolytic blister formation due to mechanical trauma (18). EBS is characterized by fragility of the skin that results in severe non-scarring blisters, and is comprised of three major subtypes and various rare types all with autosomal dominant inheritance (20, 24). The prevalence of EBS is rare with an estimated frequency between 1:30,000 and 1:50,000 individuals in the U.S. population (21).

The Dowling-Meara type of EBS (EBS-DM) is the least common and the most severe subtype with generalized blistering of the entire body. Viewed through electron microscopy, patients affected with EBS-DM are characterized by the presence of large cytoplasmic clumps of tonofilaments in the basal layer keratinocytes (2,11,13). Large, hemorrhageous blisters are present from birth, and can result in constant infections, a failure to thrive, and neonatal death (24).

The Koebner type of EBS (EBS-K) is less severe, but still expresses generalized blister formation of the entire body including mucous membrane blistering of the oral

cavity (8). Koebner EBS is present at birth and increases in severity in warmer climates. The absence of basal layer keratinocyte clumping in ultrastructural analysis helps to distinguish EBS-K from EBS-DM (4).

The final subtype is Weber-Cockayne type EBS (EBS-WC). EBS-WC is the most common and the mildest subtype usually localized to blistering of the hands and feet (3). The blistering of the hands and feet, which is adversely affected by warm climates, usually begins when a child has learned to walk or crawl lengthy distances (20). Compared to Dowling-Meara and Koebner EBS, Weber-Cockayne EBS requires more substantial trauma to produce blistering. Also, like EBS-K, EBS-WC is characterized by an absence of keratinocyte clumping in the basal layer of the skin (4).

This paper will discuss EBS through the cell biology of the skin, elucidating the proper functions and locations of various skin structures that are important in the expression of EBS. In addition, the genetic basis of EBS will be described from the initial methodology that enabled researchers to determine the genes responsible and their chromosomal locations. The importance of mutational location and amino acid substitution will be highlighted to differentiate between the three main EBS subtypes. Finally, the disheartening discussion of the current diagnosis and treatment of EBS will be offset by the optimistic search for a cure that may be obtained through the use of gene therapy.

Skin Structure and Function

The skin is a complex, multilayered organ, which produces several specialized structures called appendages such as hair follicles, eccrine sweat glands, sebaceous glands, and aprocrine glands (12). Variation in the skin is expressed by regional

differences in skin thickness, composition, and density due to the regional requirements of the skin. Thus, the palms and feet have a thick durable keratin layer, while the face and neck have a relatively thin and pliable keratin layer (12).

There are four main layers of the skin - the epidermis, the basement membrane, the dermis, and the hypodermis (5,9,12,22). Interaction among these layers is important during development and for maintaining homeostasis in the adult. In many respects, the skin layers can be best regarded as a single functional tissue and must not be regarded as independent structures (10). However, each layer will be addressed separately to emphasize and identify specific skin cell function and location.

The epidermis is the most complex skin layer and is a stratified, continually renewing epithelium that is able to differentiate (12,22). The epidermis has a tough and resilient structure that is normally able to withstand severe physical and chemical trauma. Also, it serves to keep harmful bacteria and microorganisms out to guard against infection, and helps to retain fluids to prevent dehydration (7). Cells that reach the body surface are dead, enucleated, flattened cells that are shed and replaced by inner cells that are continually moving upwards. Therefore, the epidermis is in a constant process of self-renewal, replenishing itself approximately every 2-3 weeks (7). However, if the epidermis is badly damaged by burns, wounds, or severe cuts it may be unable to replenish itself. This emphasizes another important function of the epidermis, the process of wound healing and repairs (7).

The epidermis is divided into the following zones: the basal layer, the spinous layer, the granular layer, the transitional layer, and the stratum corneum (22). In addition,

the epidermis is comprised of the following cell types that have embryonic origins: keratinocytes, melanocytes, Langerhans cells and Merkel cells (5).

The basal cell layer is the lowest layer of the epidermal cells, which borders the basement membrane (12). Basal cells, consisting primarily of keratinocytes, are the proliferating cells of the epidermis, which after a limited number of cell divisions terminally differentiate and move towards the skin surface, as more basal cells are produced. Basal cells qualify as a type of stem cell because they are able to "produce both daughter stem cells and cells that go on to differentiate to multiple cell lineages"(7). During wound healing, the rate of proliferation of these stem cells is increased and migration to the skin surface is accelerated. Columnar in shape, basal keratinocytes contain keratin 5 (K5) and keratin 14 (K14) intermediate filaments providing a cytoskeleton that has sufficient flexibility to allow cell division and migration (15). Hemidesmosomes are proteins that are important structures in cell adhesion binding keratinocytes to the basement membrane. This relationship is one part of epidermal structural integrity creating support and adhesion that prevents skin fragility and blister formation between the basal keratinocytes and the underlying dermis (22).

The spinous layer (also called the prickle cell layer) is named after the appearance of the junctions between adjacent keratinocytes resulting in the formation of 'spines' due to the flattening of the cell and a decrease in the size of the cell nucleus (9). The location of the spinous layer is directly on top of the basal cell layer, towards the surface of the skin. A particular feature of the spinous layer is the effective cohesion between adjacent cells due to intercellular bridges. Spinous cells contain bundles of keratin filaments organized around the nucleus and joined by desmosomes that cover the cell surface and

provide lateral cell adhesion (21). In addition, K5 and K14 are present in the spinous layer differentiating to keratin 1 and keratin 10 proteins as they migrate upwards from the basal cell layer (16).

The granular layer is located on top of the spinous layer in the epidermis and is characterized by the loss of the keratinocyte cell nucleus and the appearance of cytoplasmic basophilic 'keratohyalin granules' (5). These granules are comprised of dense proteins- profilaggrin and keratin intermediate filaments. The loss of keratinocyte nuclei during keratinization, a process due to the natural maturing of keratinocytes which is characterized by the increasing levels of keratin in the cells while they migrate to the skin surface, is due to destructive enzyme action (21). Therefore, the granular layer presents an area of great hydrolytic activity, which lyses most of the cytoplasmic contents prior to the formation of the stratum corneum.

The transitional layer is an abrupt transition from the granular cell to the terminally differentiated cornified cell (9). Several degradative enzymes have been isolated that are involved in the destruction of cellular organelles. The granular cell not only synthesizes, modifies, and cross-links proteins involved in keratinization, but also initiates its own programmed destruction (5).

The final layer of the epidermis is called the stratum corneum or the keratin layer. This layer is comprised of multiple layers of non-viable, terminally differentiated corneocytes that differ greatly in thickness due to regional variation, sex, age, and disease (7). The palms and the soles are the thickest regions of the keratin layer providing increased mechanical and chemical protection. In addition to providing protection, the stratum corneum is the major skin barrier to water loss and permeability of foreign

substances. Corneocytes are the largest of the keratinocytes and exhibit a flattened shape that helps provide the barrier function of the skin (5). There is a change in the structure, composition, and function of corneocytes as they move towards the surface of the skin. For example, the deep layer of the stratum corneum has corneocytes that are thicker and more densely packed creating a decreased capacity to bind water. Proteolytic degradation of the desmosomes between the outermost cells contributes to the loss or shedding of the skin (5).

In addition to the different cell layers, various cell types are encompassed in the epidermis. The predominant cells of the epidermis are the keratinocytes, which are synthesized in the basal cell layer (7,12,21). Keratinocytes are cells that produce high levels of keratin and are represented throughout the layers of the epidermis in different stages of keratinization (21).

Melanocytes are melanin producing, dendritic cells that assume a position in the basal layer of the epidermis (5). Lacking intercellular bridges, melanocytes do not form junctions with keratinocytes, which allows them to extend towards the dermis. The function of melanocytes is to produce melanin in packages called melanosomes which transfer pigment to keratinocytes giving skin its color (5).

Langerhans cells are dendritic and the antigen-presenting cells of the epidermis (5). They are responsible for the uptake, recognition, processing, and presentation of soluble antigen to sensitive T lymphocytes. Langerhans cells are important in maintaining proper immune function in the skin (12).

The fourth cell type of the epidermis is a Merkel cell. Merkel cells are intraepithelial cells that differentiated from keratinocytes (9). Epidermal Merkel cells

produce nerve growth factor and dermal Merkel cells express nerve growth factor receptors contributing to the development of the skin's nervous system (5).

The epidermis is non-vascularized and receives its nutrients from blood vessels in the underlying dermis (21). However, the epidermis and the dermis are separated by a basement membrane that is composed of more than 50 different proteins (13). The extracellular matrix proteins, collagen IV, fibronectin, and laminin 5, are the main proteins of the basement membrane (7). Both the epidermis and the dermis contribute to the cells of the basement membrane, but the basal cells of the epidermis contribute all of the collagen IV and laminin 5 cells. The basal cells of the epidermis synthesize and adhere to these cells as well as organize them into the basement membrane. Integrin is a transmembrane protein that functions in cell-to-cell interactions and adhesion (5). Integrin is an importance component of the epidermis because it acts to bind the basement membrane by the formations of hemidesmosomes that link the keratin intermediate filament cytoskeleton (9). Therefore, integrin provides great mechanical strength and strong attachment to the basement membrane. An example of the functions of integrin and hemidesmosomes are seen in the skin disorder junctional epidermolysis bullosa (JEB), which causes devastating blister formation because of a loss of adhesion between the epidermis and the basement membrane (7). In addition, the expression of integrins and the attachment of basal cells to the basement membrane are required for keratinocytes to proliferate (13).

Basal epidermal cells adhere to the basement membrane and to each other by two different structural mechanisms. Cell adhesion to the basement membrane is provided through specialized calcium-activated adhesion plaques called hemidesmosomes (25).

Hemidesmosomes contain the anchoring proteins integrin, BPAG2, and BPAG1e. The antigen for the hemidesmosome antibody is plectin, an intermediate filament linker protein (4). Basal epidermal cells interact laterally and suprabasally with neighboring cells through calcium-activated membranous plaques called desmosomes. Desmosomes are composed of distinct proteins, including desmogleins and desmocollins. The cytoskeleton network of keratin filaments is formed through the attachments to hemidesmosomes and desmosomes. These two adhesion plaques are relevant to the study of EBS because studies have shown that severing the connection of the keratin filaments to the basement membrane or to each other can result in EBS phenotypes (4).

Below the basement membrane layer of the skin lays the dermis that provides pliability, elasticity and tensile strength to the skin (9). Maintaining a strong inter-relationship, the dermis relies on the epidermis for its protection against the external environment, and the epidermis, which is non-vascularized, relies on the dermis for its nutrition and oxygen supply (7). Additionally, the dermis and the epidermis work together in repair and remodeling of the skin during wound healing. The specific functions of the dermis include protecting the body from mechanical energy, binding water, aiding in thermal regulation, and housing receptors of sensory stimuli (5).

The dermis is less cellular than the epidermis, being composed of fibrous and amorphous extracellular matrix surrounding the appendages (such as hair follicles), neurovascular networks, sensory receptors and dermal cells (9). There are two main types of connective tissue that are found in the dermis: fibrous and non-fibrous. Collagen and elastic tissue are the main types of fibrous tissue and filamentous glycoproteins, proteoglycans, and glycosaminoglycans are the three main types of non-fibrous tissue.

Collagen is the major constituent of the dermis and the skin accounting for approximately 77% of the skin's dry weight and providing necessary elasticity and tensile strength (9). There have been more than 20 different collagens identified, each composed of three chains that may be the same or very distinct from each other. However, the majority of the collagens are types I, III, and IV.

Elastic fibers are the second form of fibrous tissue and are involved in restoring skin to its normal configuration after it has been stretched or deformed. It is a continuous network of tissue that extends from the lamina densa throughout the dermis and into the connective tissue of the hypodermis.

The non-fibrous glycoproteins, glycosaminoglycans, and proteoglycans of the dermis surround the fibrous tissue and provide a gel-like milieu in which cells can migrate (5). These fibers help to regulate the water-binding capabilities of the dermis and regulate dermal volume and compressibility. In addition, through binding growth factors and cytokines the non-fibrous tissue acts to influence proliferation, differentiation, tissue repair and morphogenesis (22).

The primary cell type of the dermis is the fibroblast, a cell that migrates through the tissue and is responsible for the synthesis and the degradation of fibrous and non-fibrous connective tissue proteins (5). The ability of fibroblast cells to differentiate imparts great importance in their regulation because of increased proliferation and synthetic activity in wound healing and during the formation of scares.

The dermis is divided into two distinct regions: the upper papillary dermis and the lower reticular region. The difference in the two dermal regions is based on connective tissue organization, cell density and nerve and vascular patterns (9). A layer of vessels,

the subpapillary plexus, marks the boundary between the papillary and reticular regions of the dermis.

The upper papillary dermis is the thinner region comprised of bundles of collagen fibrils and elastic fibers. It is the outermost portion of the dermal connective tissue attaching to the basement membrane via anchoring fibrils such as collagen VII, and conforms to the epidermal ridges and grooves of the epidermis (9). The loose distribution of fibrils and the large amount of interfibrillar gel allows the papillary dermis to accommodate for mechanical stress. Also, the high density of fibroblasts in this region allow for a high rate of cell proliferation and metabolic activity (9).

The lower reticular dermis is the largest component of the dermis and the skin (13). It is constituted by large collagen and elastic fibers that form a dense layer with little vascular tissue and less fluidity. The mature elastic fibers form a structure around the interwoven strands of collagen fibers. The two fiber systems are integrated, providing the dermis with strong mechanical support. Progressively, the collagen and elastic fibers increase in size and density as they approach the hypodermis (5).

The final layer of the skin is called the hypodermis, composed of adipose tissue, which functions to insulate the body, store energy supplies, and cushion and protect the skin (5). It also has a cosmetic effect in molding to body contours. The deep boundary between the dermis and the hypodermis is defined by the transition from fibrous to adipose connective tissue.

Epidermal Keratins

Previous studies have shown that EBS is caused by a functional abnormality in the basal cell layer keratin proteins (3,19,20). Therefore, it is important to understand the

normal structure and function of epidermal keratins in order to comprehend how the keratin gene mutations cause abnormal structure and subsequent protein function leading to the skin disorder EBS. The cellular architecture of the epidermis is comprised of keratins that are essential for protective barrier function (7). A comprehensive approach to keratin proteins will be taken with a focus on the keratin 5 and keratin 14 proteins located in the basal cell layer of the epidermis.

Keratins make up the intermediate filaments, which have a diameter of 8-10 nm, that are the ubiquitous components of the cytoskeletons of epithelial cells (16). They form a class of resistant, insoluble proteins that are found in epithelium, hair, feathers, claws, and horns (15). The term keratinization is given to the process that causes these tissues to become tough and insoluble. In the human species, 30 different types of keratin protein chains are known to be expressed (13,23).

Keratins have been classified into two types based on their isoelectric points (pH 4.5-7.5): acidic (type I) and basic (type II). The keratins of one type are more closely related to each other than to keratins of the other type (2,15,23). Also, intermediate filaments are formed as components of both types being paired together based on molecular size (40-70 kDa) (11,15). On the basis of the amino acid sequence, the small (40-56.5 kDa) and relatively acidic keratins form the type I class and the larger (53-67 kDa) and more basic keratins form the type II class (2,16,26).

Amino acid sequence analysis has helped to elucidate that keratin filament assembly involves a three-chain composition (9). The primary structure of a keratin is the central alpha helical rod domain made up of seven amino acid repeating units (2). This central region of approximately 310 amino acid residues is not continuous,

consisting of four alpha helical domains that are linked to each other by three regions of beta turns, which are helix disruptions (16,23,26). These linker segments are non-alpha helical regions that are not highly conserved. The alpha helices are highly evolutionarily conserved sequences of the keratin proteins (6). The alpha helical domains of the intermediate structure are involved in the formation of the coiled-coil structure of epidermal keratins and are two-stranded structures composed of equimolar amounts of one type I and one type II keratin protein. Biochemical studies have shown that the polypeptide chains in each heterodimer are parallel and in exact axial register providing support for the two-stranded alpha helical structure (16). Intermediate filaments are formed from parallel, coiled-coil dimers, and more than 10,000 of these dimers are needed to make each 10-nm filament (16). Dimers associate in a head-to-tail fashion to make linear arrays that pack into antiparallel chains called protofilaments (2-3nm). Protofilaments intertwine to form protofibrils (4.5nm), and approximately four of these constitute the overall 10-nm diameter of the intermediate filament (16). Intermediate filaments are named because of their 10-nm diameter is intermediate between that of microfilaments (6-nm) and microtubules (23-nm) (6).

Keratin intermediate filaments provide a flexible intracellular network that functions to provide cell shape and structure and to resist stresses applied by external forces (6). Mutations that weaken the structural framework provided by keratin intermediate filaments increases the risk of cell rupture and the formation of blisters.

Keratins, like all proteins, have an amino terminal and a carboxyl terminal. The degree of homology within the central helical domains of all keratins of a single type implies that all of the interior coiled-coil regions are similar and that the differences in

keratin intermediate filaments must reside in the heterogeneous amino and carboxyl termini (15). The termini in basal epidermal keratins are rich in glycine and serine giving rise to a flexible cytoskeleton required for dividing and metabolic activity. This differs from the disulfide bond cross-bridges that are formed in cysteine-rich termini of hair and wool keratin filaments (6). In addition, differences in keratin intermediate filaments are found in the molecular weight of the carboxyl and amino termini. The different regions of keratin intermediate filaments are assigned domain titles (16). The amino terminal sequences are called the 'head' region, and the carboxyl terminal sequences are called the 'tail' region. The four alpha helices are designated as the 'rod' region named in sequential order of the amino acid sequence: 1A, 1B, 2A, and 2B (19). The three non-alpha helix regions are termed 'linker' segments and are designated L1 (2.5nm), L12 (1.6nm), and L2 (0.8nm), respectively (2,11).

Not only is there a pronounced difference in weight and length of these nonhelical termini segments of different intermediate filaments, but there is also tremendous variation in the amino acid end sequences. Even in terminal sequences of two intermediate filaments of the same type, there is little homology due to numerous gaps and insertions (16). Thus, the major differences in keratin intermediate structures of all types are found in the nonhelical terminal regions and not in the central alpha helices. Analysis of intron sequence position in the keratin genes shows conservation over millions of years of evolution and must indicate that there are strict constraints against varying the lengths of the internal exons of the intermediate filament genes (16). Homology between different intermediate filaments is very high, especially at the beginning of helix 1A and at the end of helix 2B.

The degree of homology of intermediate filament rod domains emphasizes the importance of the linker regions in forming the coiled-coil dimer, and in recognizing their perspective partners in dimer and tetramer formation (23). Deletions in the linker regions do not prevent dimer and tetramer formation, but they do interfere with filament stability and proper keratin function.

The diversity of the head and tail regions contribute to the differences in keratin filaments and are tailored to suit a particular 10-nm filament network. One general role shared among the tail domains of keratins is to stabilize protein-protein interactions between intermediate filament subunits. However, the role of the head domain is more complex playing a role in the spartial alignment of subunits during intermediate filament assembly. In addition, portions of the head and tail regions extend along the surface of intermediate filaments interacting with various cell structures and organelles (6).

Studies Leading to the Genetic Basis of Epidermolysis Bullosa Simplex

Early ultrastructural studies of patients with severe blistering found an unusual clumping of keratin intermediate filaments that preceded cell cytolysis and blister formation (13). Also, studies showed that shortened keratin filaments led to a disruption of the keratin network and intermediate filament assembly (8). Since clumping of keratin filaments preceded blister formation and cytolysis, it was postulated that EBS was likely to arise from structural defects in the epidermal keratins. The most important evidence indicating that keratin mutations were responsible for blistering disorders came from a reverse genetics experiment where mice were introduced with a mutant keratin K14 gene into their germ line. The transgenic mice exhibited nearly all of the phenotypic and biochemical traits characteristic of EBS-DM, including mechanical stress induced

blistering over the body surface, clumping of keratin filaments, and cytolysis of the basal layer cells (2,25). In mice, the human keratin gene was appropriately expressed in the basal layer of the epidermis and other stratified squamous epithelia. Transgenic mice were important in identifying both the keratin K14 gene and the keratin K5 gene as being responsible for EBS. The mutation in transgenic mice caused aberrations similar to those in cultured EBS keratinocytes demonstrating that the mutations in K14 were responsible for the disease (3). In addition, strategically engineered mutations in various positions on the K5 and K14 genes indicated the relationship between mutation location and the severity of the disorder (4). When transgenic mice were engineered with mutations that expressed low-level keratin mutations, observed phenotypes were more similar to EBS-WC with blistering predominately on the paws. In the same fashion, when severe disruption of keratin filament assembly was induced, phenotypes were observed that corresponded closely to EBS-DM, with total-body trunk blistering (4).

Another study that helped researchers determine the genetic basis of EBS involves genetic linkage analysis (1). Families affected with EBS were studied for inheritance of keratin intermediate filament abnormalities. The researchers found that the inheritance of EBS was linked to keratin genes K5 and K14 (1).

Unequivocal evidence that human EBS can arise from genetic defects in K14 and K5 came from sequencing the corresponding genes from normal patients and EBS patients and from conducting mutational functional analysis of the defects. A number of point mutations in either of the keratins K5 or K14 were identified. As expected from the heterodimeric nature of the basal layer keratin filaments, EBS can arise from defects in the K14 or K5 genes (1).

Genetic Analysis of EBS

EBS is caused, in most cases, by missense mutations in the K5 and K14 genes (10). More than 50 different keratin mutations have been discovered highlighting the existence of mutational "hotspots" (14). Keratin proteins that have sequence changes in these hotspot regions that encode the helix initiation and termination sequences are more likely to have detrimental protein function. Mutations that occur outside these hotspots are usually less severe disease phenotypes, such as EBS-WC (11). However, some studies have observed nonsense mutations that resulted in premature stop codon mutations of K14 and K5 (10). One example of a mutation that resulted in the expression of EBS-DM was observed in a patient that carried a heterozygous A to G transition at nucleotide position 368, changing asparginine to serine at codon 123 (22).

Once the keratin 5 and keratin 14 genes where identified as the underlying cause of EBS, restriction fragment length polymorphisms (RFLPs) were used to link EBS to the keratin gene clusters on chromosome 12 and chromosome 17 (3). Linkage analysis and improved markers demonstrated that the genetic defect in EBS mapped to the locations of known loci for epidermal keratin type I and type II clusters. The keratin 5 gene was determined to reside in the chromosome 12q11-q13 region, and the keratin14 gene was isolated to the chromosome 17q12-q21 region (3,4,24).

There is a strong correlation between the severity of the EBS disease, the location of the EBS mutation with respect to keratin structure, and the degree to which a particular mutation disrupts filament assembly. EBS-DM and EBS-K are characterized by mutations that occur in the conserved alpha helical regions of the keratin proteins. In particular, EBS-DM associated mutations reside in the highly conserved ends of the 1A

or 2B segments, whereas EBS-K associated mutations are usually located more centrally in the less conserved rod domains. EBS-WC mutations are most frequently in the non-conserved regions such as the linker region L12, in the head region of K5, or in the 2B segment of K14 (22).

There is a strict genotype-phenotype correlation in EBS, whereas the same mutation will always result in a single phenotype (22). The location of the keratin mutation is important, yet it is not possible to predict the resulting phenotype merely by the position of the mutation. The other critical factor in determining the severity of the EBS phenotype is the nature of the amino acid substitution (10). For example, studies have shown how two different mutations in the same location of the keratin gene result in drastically different functional consequences on the keratin intermediate filament. For instance, differences may arise because one mutation resulted in a hydrophobic amino acid substitution and the other mutation resulted in a hydrophilic amino acid substitution altering the arrangement of the keratin intermediate filament (22).

The autosomal dominant mode of inheritance characterized in EBS arises because mutant keratins are able to recognize their obligatory heterotypic partners and act in a dominant negative fashion to disrupt keratin network formation. For instance, a person affected by EBS-WC receives a normal allele from the unaffected parent and a mutated allele from the affected parent. Autosomal dominant diseases are not sex-linked and affected parents have a 50% chance of passing on the genetic mutation to their offspring.

The cytolysis of basal cells is linked to the lack of a proper keratin filament network. Without the cytoskeleton structural support of the keratin intermediate filaments, basal cells become fragile and are prone to breakage upon mechanical stress

(4). Functional demonstration of this has come through studies of mice and humans that have a null mutation in K14 and lack a typical basal cell keratin network (2). Since K5 and K14 form obligatory heterodimers, in the absence of K14, K5 cannot assemble into keratin filaments. However, K5 is present but in very low abundance forming wispy filaments within the basal epidermal cells. Interestingly, the patients and the mice lacking K14 expression have less severe blistering than those with severely disrupting dominant-negative mutations, suggesting that aggregates of insoluble keratin worsen the cell degeneration in EBS cases. In EBS-WC cell rupturing occurs in a defined zone, beneath the nucleus and above the hemidesmosomes, which is within the region of greatest structural strain where the cytoskeleton spans the distance between the nucleus and the plasma membrane (21).

Unique Features of EBS

A unique feature of EBS-WC is that blisters generally heal without scarring. In mice studies, it has been shown that during wound-healing, basal cells flatten, reduce their levels of K5 and K14 synthesis, and do not lyse (4). These results support the idea that lysis is a mechanical trauma-dependent phenomenon that is likely to occur from a compromise in mechanical integrity, and that mechanical stress may be greater when cells are columnar than when cells are flattened (4).

Research has indicated that EBS-WC worsens in hot climates especially during the humid summer months (3,4,17). The increased blister formation could be due to temperature-sensitive changes in the mechanical integrity of basal cells, particularly because of their high level of fluidity. It is also known that induction of heat-shock proteins can induce alterations in keratin filament networks in simple epithelial cells. In

addition, the stability of the keratin network or its interactions with other cellular components could change in a temperature dependent fashion.

One of the most interesting features of EBS-DM and EBS-K is that the phenotypic expression of the disease improves with age (4,17). There are several possible theories to explain this feature, but the most likely explanation is that mechanical stress on basal cells is the greatest during development. In neonatal skin, the cellular anchors of the epidermis are much smaller, and the support structures are not fully formed. Therefore, maturation is likely to improve the mechanical strength of the skin, lessening basal cell fragility (4).

Diagnosis and Therapy of EBS

Methods for diagnosis of EBS are very reliable, and often a preliminary diagnosis can be made after a routine patient exam. Easy blistering at birth or in childhood due to mild mechanical trauma and the healing of wounds without scarring are signs that a patient might have EBS (1). However, for an accurate diagnosis ultrastructural analysis of a skin biopsy from a lesion is required. The ultrastructural analysis will reveal intraepidermal cytolysis of the basal cell layer without perturbations in the suprabasal layers. In addition, EBS-DM is distinguished through clumping of the keratinocytes, which is not seen in EBS-K and EBS-WC (11).

Visual analysis is still used to distinguish between the mildest and most severe EBS subtypes. EBS-WC is largely restricted to the hands and feet whereas EBS-DM and EBS-K exhibit more generalized blistering over body trunk areas. EBS-DM is more severe than EBS-K and frequently is characterized by clustered lesions and cutaneous and oral cavity anomalies (21).

The accumulation of genetic data has made it possible to develop DNA screening methods for the diagnosis of EBS and for genetic testing within an affected family. Given that most EBS mutations are clustered in the K5 and K14 rods, it is possible to rapidly identify the key mutation within most EBS families, through the use of either polymerase chain reaction (PCR) analysis and sequencing of genomic DNA or through immunologic tools to distinguish the normal sequences from the mutated sequences in the keratin genes (21). Once the location of an EBS mutation has been identified in an affected parent, it enables future prenatal diagnosis (18). As in vitro methods are developed to distinguish normal and mutant embryos, it may be possible in the future to eliminate the disease in future offspring of an EBS family.

Future Treatments of EBS

There is currently no cure for EBS and therapeutic methods have been largely ineffective. Clinical treatment of children with EBS-DM is the most challenging to physicians and family caregivers because of its debilitating effects. Great care has to be taken to prevent physical trauma and bacterial infections during the first several years of life. In most cases, sterile bandages and antibiotic ointments are used to guard against infection. Since blistering is made worse by heat, a cool environment is important.

The epidermis appears to be a feasible target for tissue for gene therapy because it is readily accessible and can be easily removed in the event of unwanted effects (13). In addition, the epidermis is continually renewing through the activity of stem cells in the basal cell layer. This creates the possibility of transducing these stem cells for a long-term treatment of patients. However, gene therapy is still a very new concept and many

problems are yet to be answered including the mode of gene delivery and controlling the differentiation of stem cells (13).

Transgenic mice studies have also been important in showing that EBS may be a disease amenable to gene therapy (4). In mosaic transgenic mice, in which the mutant human keratin gene is integrated into the mouse's chromosomal DNA after the first cell division of the embryo only a fraction of the transgenic animal's epidermal cells contained the mutant gene, while the remainder were wild type. Given these findings it appears there is a strong natural selection pressure against the mutated cells. When healthy wild-type cells are transplanted in the presence of mutant EBS cells, the healthy cells seem to rapidly replace basal layer vacancies left by ruptured EBS cells. By developing methods to engineer out or replace the mutant keratin gene in cells cultured from an EBS patient, cultured now-healthy epidermal skin grafts could replenish affected areas (4). In addition, future improvements in homologous recombination techniques may allow researchers to remove or inactivate the point mutations responsible for most EBS subtypes (13). Thus one of the problems associated with gene therapy is developing the technology for rapidly engineering out a defective keratin gene from the keratinocytes.

Conclusion

Epidermolysis Bullosa Simplex has proven to be a disease that is very debilitating, painful, and limiting of affected individuals' daily lives. However, by studying diseases such as EBS, that have a strong genetic basis and complex cell biology, researchers are able to gain a better understanding of human biology and disease. For example, analysis of the skin from a genetic and molecular perspective has underscored

the importance of cell adhesion, keratin intermediate filament structure and assembly, and tissue architecture in contributing to the proper balance, differentiation, and function of the epidermis. The combination of human biology and genetics will allow future treatments and cures to be discovered for many diseases that are presently incurable.

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