Morphogenesis and Malformations of the Skin NICHD/NIADDK Research Workshop

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Developmentally caused skin malformations constitute a spectrum of birth defects, some of which can be recognized prenatally by morphologic or biochemical means. The number of prenatally diagnosable skin diseases could be greatly expanded with an increased understanding of the molecular and cellular bases of skin development and the mechanisms that result in the generation of skin defects. The National Institute of Child Health and Human Development and the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, therefore, sponsored a workshop that recommended basic biologic studies combined with clinical investigations of normal and abnormal cutaneous development set forth in this article. Investigations resulting from these research recommendations are intended to contribute to the knowledge that should aid in the prevention of developmentally caused skin deformities.
J Invest Dermatol 88:464–473, 1987

The skin is a complex organ consisting of cells and tissues derived from ectoderm (the epidermis, its appendages, nerve networks), mesoderm (cells and extracellular matrix of the dermis, vascular systems), and the neural crest (melanocytes). Each of these tissues becomes unique in its structure, biochemistry and function during development of the skin. Each also maintains cell-specific characteristics that are expressed in other organs throughout the body. For example, epidermal cells, like all epithelial cells, contain keratin proteins; the fibrous proteins present in the dermis are also synthesized in tendon, bone, and cornea; and all basal laminae, including the dermal-epidermal junction, have certain structural proteins and antigens in common. The skin is accessible, and samples may be obtained easily for cutaneous studies specifically, and for general investigations of cell and tissue interactions and of important macromolecules that have a general body distribution.

Cell and tissue interactions are difficult to study in adult skin because of its structural and biochemical complexity. The developing skin provides a situation in which structure is achieved in a step-wise fashion. Thus, complicated events such as epithelial-mesenchymal interactions, patterning of epidermal appendages, and assembly of dermal connective tissue macromolecules may be approached more easily. The problems that may be studied in developing skin are as varied as the components of skin itself, and require the interest and talents of a diverse group of investigators.

Until the last decade, this diversity was not reflected in studies of cutaneous morphogenesis; rather, descriptive morphology and experimental embryology dominated the field. There is at present a heightened interest in cutaneous development in man. In part, this may be a consequence of several new technologies and may also be a result of a more widespread acceptance of clinical procedures for the prenatal diagnosis of inherited skin disorders. Immunologic techniques, particularly the development and use of monoclonal antibodies, have been important in evaluating the state of differentiation of normal fetal skin and in assessing developmental abnormalities in skin from fetuses and newborns with genetic skin disease. The relatively recent success in culturing epidermal keratinocytes has opened the door to studies of normal and abnormal epidermis in the same manner that fibroblast cell culture has allowed connective tissue biologists to investigate the molecular basis for inherited connective tissue disorders so successfully. The use of the nude mouse as a host for transplants of normal and abnormal human skin has provided an opportunity to investigate the site of action of an abnormal gene in the skin and to conduct epidermal-dermal recombinant studies with human tissue in a physiologic environment. The development of gene probes and the use of these in sequencing and mapping genes is at the forefront of research related to genetic diseases of the skin. All of these technologies are being used successfully to answer questions about normal and abnormal adult skin. The biology of normal and abnormal skin during development can now be approached with similar techniques.

Cutaneous morphogenesis and malformation in humans was the topic of a March of Dimes (MOD)-sponsored symposium in 1980. This landmark meeting brought investigators together to discuss skin development in man. The morphology of human skin development was reviewed, new research techniques were explained, the use of mutant strains of mice that are applicable to experimental studies of skin development were discussed, and

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Abbreviations:

APUD: amine precursor uptake and decarboxylation
BP: bullous pemphigoid
DEJ: dermal-epidermal junction
EBA: epidermolysis bullosa acquisita
EDs: ectodermal dysplasias
EGA: estimated gestational age
EGF: epidermal growth factor
MEL: mouse erythroleukemic

0022-202X/87/$03.50

the understanding of the basis for specific genetic skin disease was reviewed. Aging syndromes were included as the antithesis to development. The meeting ended with a discussion of the current options for prenatal diagnosis of skin disease and a prospective look at the potential for expanding this capability.

The MOD meeting helped to set the stage for the workshop sponsored by the National Institutes of Health (NIH) on Morphogenesis and Malformations of the Skin, which was held in September 1983. Cosponsorship of this meeting by two NIH Institutes, the National Institute of Child Health and Human Development (NICHD) and the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (NIADDK), reflected the Institutes' overlapping interests, i.e., the specific concern for skin development by NICHD, skin biology by NIADDK, and for abnormalities in development or genetic skin disease by both units.

This workshop brought together basic and clinical scientists working in the area of skin development, or in a related area that would provide an important perspective on some aspect of skin morphogenesis. Workshop participants had expertise in cutaneous differentiation; molecular structure of the skin and/or skin-related macromolecules; genetic diseases of the skin; structure, function, biochemistry, and/or physiology of the skin; and/or prenatal diagnosis. The participants were: Eugene A. Bauer, Robert A. Briggaman, Frank Chytil, Benoit de Crombrugghe, Beverly A. Dale, Delbert H. Dayton, Kjell Elgjo, Cynthia J. Fisher, Elaine V. Fuchs, Paul F. Goetinck, Lowell A. Goldsmith, Karen A. Holbrook, Barbara Johnson-Wint, Stephen I. Katz, Lloyd E. King, Anne K. Krey, Alfred T. Lane, Alan Moshell, Vincent M. Riccardi, Clara V. Riddle, Dennis R. Roop, Joel Rosenbloom, Roger H. Sawyer, Philippe Sengel, George Stamatoyannopoulos, Malcolm S. Steinberg, Peter M. Steinert, Marry Ellen Stewart, George P. Stricklin, Bryan P. Toole, Juoni J. Uitto, James A. Weston, and Sumner J. Yaffe.

The program of the workshop was necessarily broad, reflecting the diversity of topics relevant to future skin development and diseases research. Dr. Stamatoyannopoulos, of the University of Washington, was invited to be the keynote speaker because of his elegant studies on gene regulation during development. His work on hemoglobin switching provides a prototypic model for studies of the developmental control of gene activity, a topic that is particularly applicable to the skin.

The first of two sessions of the workshop, entitled "Morphology and Macromolecular Expression," concentrated on the morphology of the epidermis and its appendages, dermal-epidermal junction, and the dermis; and on the expression and regulation of structural and enzymatic proteins and lipids, in the respective regions of the skin during differentiation in the adult and in development. The topics that were discussed in the second workshop session, entitled "Control Mechanisms," included the mechanisms of tissue interactions during cutaneous development, the role of cell-cell and cell-matrix interactions, and the effects of intrinsic and extrinsic regulatory compounds (e.g., hormones, growth factors, vitamins, and steroids) in modulating skin development. Whereas these were logical categories for the purpose of presentation, it was apparent that the research that needs to be done is going to focus either on questions involving the epidermis, the dermal-epidermal junction, the dermis, or dermal-epidermal (mesenchymal-epithelial) interactions. This report will follow the latter scheme of organization.

THE EPIDERMIS

Structure of the Epidermis During Development Studies of the structure of the epidermis have revealed the characteristic features and the timing of events in the development and differentiation of fetal epidermis in utero. Our understanding of these events in adult epidermis has allowed us to look for important developmental markers. The characteristics that define the epidermis at different stages of development are: stratification, appearance of immigrant cells (melanocytes, Langerhans cells, and Merkel cells), changes in periderm structure, initiation and development of epidermal appendages (pilosebaceous structure, eccrine and apocrine sweat glands, and nails), and keratinization [1].

A feature that is unique to the developing epidermis is the periderm, a transient, simple epithelium first present in embryonic skin as the superficial layer of the two-layered epidermal tissue. The periderm is retained throughout the first two trimesters of development and undergoes a remarkable series of morphologic changes in which flattened, pavement-like epithelial cells project large, multiple blebs, then regress to a flattened epithelium [2]. Its last cells desquamate into the amniotic fluid at the time cells of the underlying epidermis proper undergo keratinization. The amniotic surface of the periderm is densely covered with microvilli, even over the blebs, indicating that this layer presents an enormous amount of surface area to the amniotic cavity—a structural feature that may have functional properties in terms of exchange between the fetus and the amniotic fluid [3]. Recent studies have shown that the periderm contains the keratin polypeptides that are characteristic of simple epithelia, and that these are retained throughout gestation, even though the underlying epidermal layers at the same time progressively synthesize new keratin species [4]. There is little more than speculation, however, as to the role of the periderm, the origin of this tissue, and its relationship to the underlying epidermis and possibly with the amnion. New monoclonal antibodies that recognize an antigen on periderm cells should be valuable in sorting out the identities of this layer as compared with other epithelia, and in noting other properties of these unique cells [5].

Markers of Fetal and Adult Epidermal Differentiation Differentiation in the adult epidermis is evaluated in terms of structural proteins (particularly keratins and filaggrin), cell surface antigens and receptors, proteolytic enzymes (e.g., plasminogen activator) and epidermal lipids that are expressed.

Structural Proteins

KERATINS There has been significant progress in understanding the structure and biochemistry of keratin proteins in the epidermis and other epithelia both in vivo and in vitro. Genes for some of the keratins in mouse and human epidermis have been sequenced, and cDNA probes that can hybridize with mRNAs from tissue and cultured keratinocytes have been prepared.

The keratins are a multigene family ranging in apparent molecular mass (M_r) from 40,000 to 68,000 (40–68 K). Most of the keratins are products of distinct mRNAs [6,7]. A few (the 65 and 66 K keratins) are the result of posttranslational modification of the 67 K keratin [8]. There is considerable evidence for posttranslational modification in mammalian epidermis [9,10], but the mechanism is not understood. The keratins belong to either an acidic (I) or basic (II) subfamily and are found with other members of their group in the same keratin intermediate filament [11]. The molecular organization of keratins in keratin filaments has been inferred on the basis of amino acid sequence and structure of certain keratin genes [12], and confirmed by scanning transmission electron microscope studies [13]. Our present understanding of keratin filament expression by, and the filaments' localization in the epidermis has thus relied largely on protein chemistry, cell-free translation of mRNAs from tissue, and monoclonal antibody studies [14–16].

Studies using cultured keratinocytes have also suggested that the various keratins are products of distinct mRNAs [6]. Culture systems have been particularly important in the demonstration of environmental effects on keratin expression and the modulation of epidermal differentiation in the adult. Cultures of fetal keratinocytes have not been used in such studies.

Keratin expression by epidermal keratinocytes depends upon the state of differentiation of these cells. In adult epidermis, keratins of increasing M_r are synthesized as cells migrate from the basal cell layer into the stratum granulosum and stratum corneum.

The 50 and 58 K keratins expressed by basal keratinocytes, and the 56.6 and 67 K keratins expressed by suprabasal cells are considered markers of epidermal stratification and keratinization [17].

A similar sequence of keratin expression occurs in fetal epidermis, with progressive stages of gestation and concomitant epidermal stratification [4]. At the time of the embryonic-fetal transition, a layer of intermediate cells is added between the basal and periderm layers. These intermediate cells start synthesizing the high-M_r keratins around 11 to 14 weeks, when hair follicles begin to form and initiate keratinization. Posttranslationally modified keratins are expressed around 24 weeks, after epidermal keratinization is complete. At the same stage, 40 and 52 K keratins expressed uniquely by the fetal epidermis are lost, probably due to desquamation of the periderm or masking by other keratins. The sequence of keratin expression suggests that new genes are activated at different developmental stages, with perhaps continuing expression of the keratin genes from the earlier stages. The knowledge of keratin expression by embryonic and fetal epidermis provides a rational basis for the diagnosis of disorders that involve abnormalities in keratin filament structure and biochemistry expressed in utero sufficiently early to be recognized by prenatal diagnosis based on amniocentesis and/or fetoscopy.

The normal pattern of keratin filament expression is altered in certain keratinization (e.g., some of the ichthyoses) and hyperproliferative (e.g., psoriasis [18]) disorders affecting the epidermis. Among these alterations are an absence of expression of some of the tissue-specific keratins, the reexpression of embryonic and fetal keratins, and the expression of "new" keratins. The 48 and 56 K keratins have been described as hyperproliferation markers [19]. Such markers need to be studied.

Keratin expression can be modulated by hormones during normal physiologic events (e.g., cyclic changes in vaginal epithelium), by addition of exogenous hormones to culture systems (corticosteroids), by various pharmacologic factors (e.g., retinoids), and by components of the dermis (dermal-epidermal interactions).

The hormonal regulation of keratin expression has been studied using cultured mouse epidermal cells and the hormonally-regulated cyclic differentiation of the rat vaginal epithelium. Estradiol administered to ovariectomized mice induces differential expression of keratin genes in a temporal sequence. Twenty-four h after the hormone is administered, the epithelial cells begin to proliferate, and genes for the 50, 55, and 60 K keratins are expressed. After 48 h, the epithelium is stratified and keratinized, and the cells express the higher-M_r keratins. cDNA probes have been prepared that hybridize with mRNAs of differentiated mouse epidermal keratins (M_r 55, 59, and 67 K) and to mRNAs for low-M_r keratins (M_r 50, 55, and 60 K) [16,17]. The use of similar probes in studying human skin development would be valuable for correlating the expression of keratin genes with the timing of onset of synthesis of the specific protein markers.

The influence that hormones (or other substances) in the amniotic fluid or circulating in the fetus might have on keratin expression in the human fetal epidermis has not been investigated to date. It is known that the amniotic fluid is high in steroid hormones (estrogens, etc.) and that fluctuations in the levels of these substances in other body fluids influence, at least, the structure of the epithelia (e.g., the epithelium of the third ventricle) they bathe. It is intriguing to consider that there may be a similar influence on the developing skin, and that hormones might affect the expression of keratin genes.

Avian systems have been particularly valuable in studies of the pharmacologic control of the epithelial differentiation (keratin polypeptide expression). Most of the efforts in these systems have been concentrated on studies of dermal-epidermal interactions, and will be discussed separately below. Other studies have involved injections of hydrocortisone and triamcinolone into chick embryos at different stages of development. The result of this treatment is an inhibition of scale formation, because β keratin synthesis is blocked in the steroid-treated embryos [20]. It is not known if, or how, fetal skin in humans binds and responds to corticosteroids that may be present in the fetal circulation or in the amniotic fluid.

The relationship between vitamin A metabolism and the promotion and maintainence of keratinization in the skin has long been recognized. However, the mechanism of action of the vitamin A–derived substances (retinoids), their physiologic role, and the application of this knowledge to clinical practice have been rather recent developments. Vitamin A added to keratinocyte cultures inhibits the expression of high-M_r keratin genes, but permits terminal keratinocyte differentiation if it is deleted from the culture medium [21]. The action of vitamin A (retinol) and retinoic acid may be mediated by two distinct intracellular binding proteins, which have been purified to homogeneity and characterized. Both proteins are found in all animals, bind retinoids in a species- but not tissue-specific manner, are expressed at different stages of differentiation, and have been identified in fetal chick skin extracts [22]. They may be the second messenger system that can activate and repress the genome at different stages of differentiation [23]. In spite of the well-known teratogenicity of retinoids for a number of fetal organ systems [24] and their influence on the differentiation of the epidermis, there has been no effort to determine if the intracellular retinol or retinoic acid binding proteins are present in human fetal skin. The presence of these molecules may mediate the retinoids' influence on cutaneous morphogenesis.

FILAGGRIN Filaggrin and its precursor, profilaggrin, are proteins found in the highly differentiated epidermal layers, granular and cornified. Profilaggrin is a highly phosphorylated, polymeric, basic protein found in keratohyalin granules [25]. It is converted by dephosphorylation into tri-, di-, and monomeric units of filaggrin during the granular-cornified cell transition [26]. Filaggrin molecules are broken down to free amino acids, urocanic acid, and pyrrolidone carboxylic acid in the outer layers of the stratum corneum [27]. Recent studies combining protein biochemistry, immunohistochemistry, and electron microscopy have shown that filaggrin is expressed in keratinocytes of fetal epidermis only when there is morphologic evidence for granular and cornfied cells. This occurs around 14 weeks estimated gestational age (EGA) at the site of developing hair follicles, and approximately 10 weeks later in the interfollicular epidermis [4]. Thus, this marker of terminal differentiation of the epidermis appears significantly later in development than the high-M_r keratins. This is an unexpected finding, because it is known that there is coordinated expression of profilaggrin and the high-M_r keratins in vitro [28]. The factors that initiate the expression of filaggrin are unknown.

DESMOGLEINS AND DESMOPLAKINS Keratinocytes in all layers of the embryonic epidermis, including the periderm, are connected by desmosomes, the protein components of which are important for the integrity of tissue structure. Desmosomal proteins may be involved in some of the acantholytic disorders of the skin, such as Hailey-Hailey's disease and Darier's disease. Two groups of proteins have been recognized: those that occur extracellularly (desmogleins), and those that are found in the cytoplasmic environment (desmoplakins). These proteins have been isolated, characterized by M_r, used as antigens to produce both polyclonal and monoclonal antibodies, and localized in the epidermis by immunoelectron microscopy [29,30]. Three monoclonal antibodies produced against one of the desmogleins also react with the α-2 type 1 collagen band, suggesting that there may be a collagen-like region in the desmoglein molecule. Only 2 of the 5 monoclonal antibodies against desmosomal proteins recognize proteins of the hemidesmosome, indicating that these connections to the basal lamina are, in spite of their morphologic similarity to desmosomes, biochemically distinct.

OTHER PROTEIN MARKERS OF DIFFERENTIATION Involucrin is a protein marker of epidermal differentiation produced in soluble form by cells in the spinous layer, but crosslinked in the granular

layer cells by the calcium-requiring epidermal transglutaminase [31]. The crosslinked protein assembles beneath the plasma membrane of cornified cells as a thin, electron-dense layer that, together with the plasma membrane, forms the cornified cell envelope. The regulation of involucrin expression has been examined in keratinocyte culture. Postmitotic keratinocytes that either remain in or have left the basal layer synthesize involucrin [32]. Involucrin is expressed in developing rabbit skin only after the epidermis has stratified [33]. Involucrin synthesis by human fetal epidermal cells has not been investigated to date.

Cell Surface Antigens and Receptors: Several antigens present at the surface of keratinocytes appear to mark the state of keratinocyte differentiation. For example, the glycoconjugates on keratinocyte surfaces of different layers of the adult epidermis [34], and on keratinocytes in vitro [35] bind lectins differentially, corresponding to a loss of carbohydrate moieties as differentiation proceeds. Furthermore, blood group antigens are expressed differently by fetal epidermis and oral mucosa cells [36], but it is not known how these antigens, or the various lectins label the epidermis during prenatal development in utero.

Various cellular receptors may be essential to keratinocyte differentiation. For example, the epidermal growth factor (EGF) receptor has been isolated and purified, and an antibody has been prepared to it that appears to bind preferentially to the least differentiated cells in the epidermis and in keratinocyte cell cultures [37–39]. Localization of the EGF receptor has been studied in normal and diseased skin and in cultured keratinocytes, and EGF activity has been determined therein. EGF exerts its growth-regulating effects by binding to the EGF receptor, which may be the same molecule as the EGF kinase and major endogenous membrane protein; subsequently, it is internalized and rapidly degraded. The element(s) of this regulatory mechanism that stimulate cell proliferation are not known, but one signal may be the increased phosphorylation of tyrosine residues of endogeneous and exogenous proteins, which occurs when EGF binds to its receptor molecule. The EGF system in adult skin is one of the best-studied models of growth and differentiation. EGF receptor distribution has also been studied in fetal rat skin, where it was found to label basal epidermal cells, except for the developing follicles [40], but the position of EGF receptors in developing human skin and the role of EGF, or of similar morphogenetic substances, in the ontogeny of this tissue has not been investigated.

Epidermal Lipids: Epidermal lipids are often neglected as markers of differentiation, although the lipids synthesized by the cells of the more highly differentiated epidermal layers are as specific for these cells as the keratins they possess. The lamellar granules present in spinous and granular cells are the source of neutral lipid–rich lamellae that are released by exocytosis between the granular and cornified cell layers [41]. These stacks of lamellae are thought to be important in regulating permeability of the adult epidermis. Lamellar granules are recognized in the keratinizing hair canals of fetal skin at around 15 weeks, and in the interfollicular epidermis just at the onset of keratinization, around 22 to 24 weeks [42]. Epidermally derived lipids are also important in promoting the desquamation of horny cells, an essential activity if the epidermis is to be maintained in a constant thickness [41].

Lipids present on the skin surface are a mixture of epidermal lipids and sebaceous gland–derived sebum. Secretion of lipids by the sebaceous gland is influenced by circulating levels of androgens, although the fatty acid composition of these lipids is influenced by both hormones and genetic factors. There is morphologic evidence that sebum synthesis begins in fetal skin at around 15 weeks of gestation and probably makes significant contributions to the lipid-rich material of the vernix caseosa that covers fetal skin, particularly in later stages of gestation [1]. It is not known if or how the vernix influences permeability properties of the fetal skin, or whether the composition of lipids in the amniotic fluid could be used as a criterion for prenatal diagnosis in some ichthyoses.

The first reported studies of fetal cutaneous lipids have shown distinct differences between epidermal and dermal lipids, and that even in the first trimester, the epidermis synthesizes some of the lipids that are characteristic of adult tissue [43]. There are insufficient data at present to correlate lipids in fetal skin with its permeability, although studies with fetal skin in vitro have demonstrated that water transport across human fetal skin declines markedly when the composition of amniotic fluid changes and the fetal epidermis begins to keratinize [44]. More recent studies of epidermal morphology by freeze-fracture techniques have shown that the plasma membranes of fetal epidermal cells have properties typical of epithelia involved in water transport during the first half of gestation [3]. The role of the epidermal lipids in the fetus' exchange with the amniotic fluid is an important problem that needs investigation.

Epidermal Stem Cells and Regulation of Epidermal Proliferation

Epidermal Stem Cells: Heterogeneity in the proliferative capabilities of basal epidermal cells has only recently been appreciated in work on rodent and avian epidermis [45,46]. Studies of adult human epidermis grafted to the nude mouse [47] have revealed a population of label-retaining cells that may be equivalent to certain basal cells in adult rodent epidermis that have properties of stem cells: they cycle slowly and retain label for long periods of time. Similar cells have also been identified in the monkey palm, where they are located rather precisely [48]. There are as yet no general morphologic or cytochemical markers that can distinguish stem cells from other basal cells in adult epidermis. Proliferation of the epidermis in the human embryo or fetus has been studied in only a few instances, and then limited in scope to a few attempts at labeling proliferating epidermal cells in organ culture [49]. There have been no attempts to identify stem cells in this rapidly growing tissue.

A challenge for the future is to improve our understanding of the kinetically different populations of basal epidermal cells and to develop markers that can be used to identify a population of cells that possess the properties assigned to stem cells. Such cells must play a role in regulating normal epidermal structure and may be target cells in hyperproliferative disorders. The establishment of embryonic and fetal skin and/or keratinocytes in organ and cell culture should provide the opportunity for studies of epidermal cell kinetics, and stem cells, in developing skin.

Regulation of Epidermal Proliferation: In epidermal as well as other cell systems, more differentiated cells have been shown to regulate the amount of cell proliferation by negative feedback. Extracts of epidermis can inhibit proliferation of basal keratinocytes at certain times in the cell cycle. This inhibition is proliferation-dependent, and requires certain cAMP levels to act. An acidic pentapeptide with the sequence pGlu-Asp-Ser-Gly-Ala has been identified as an epidermal inhibitor that influences the cell cycle after completion of DNA synthesis. This inhibitor strongly suppresses mitoses in rat tongue epithelium when administered in low doses, but has a stimulatory effect at high doses. A granulocyte inhibitor of only five amino acids has a similar inhibitory effect on cells of the bone marrow [50]. The mechanism for this inhibitory effect and the tissue specificity of the inhibitors have not been determined as yet.

Model Studies for Probes of Gene Regulation During Epidermal Development and Differentiation Significant progress has been made in understanding protein markers of epidermal differentiation, but the genes that specify form and function of the epidermis have not been adequately investigated as yet. Recent advances in analyses of molecular controls of development now

make it possible to determine the contributing factors that influence the developmental regulation of the epidermal genes. Controlling influences have been elegantly studied for the hemoglobin genes during erythropoiesis. Such investigations of relative contributions of embryonic environment and intrinsic cellular commitment to the developmental switching of hemoglobin genes are discussed below to provide a model for probes into the regulatory controls of epidermal genes during development of the skin.

Erythropoiesis begins in the yolk sac, but is taken over by the liver at approximately 5 weeks EGA, and is ultimately the role of the bone marrow, at approximately 20 weeks. Simultaneously, globin synthesis undergoes developmental changes that involve a switch (or switches) from primitive to more mature forms of globin expression. Activity of the θ gene in the embryo is replaced by expression of the two α genes in fetal and adult life. Genes of the β locus switch from expression of the ϵ chain in the embryo to the γ chain in fetal life and to the adult β chain, with onset in the fetus and an increase in the perinatal period. The θ to α, and ϵ to γ switches occur by the 5th week of gestation, when hematopoiesis changes from yolk sac to liver, with completion by 60–80 days EGA [51]. Activation of the γ and β genes at the beginning of the fetal period occur simultaneously, but γ chains are produced at a rate 50 times higher than β chains at that stage [52]. The switch from γ to β in the perinatal period is not correlated with a switch in site of hematopoiesis, but appears to correspond to the developmental age of the fetus/newborn [53]. Switching is delayed in developmentally retarded fetuses, and is not modified in sheep fetuses that have been either thyroidectomized, adrenalectomized, hypophysectomized, or nephrectomized (to test for a possible influence of hormones on the switch) [54].

Of the two hypotheses that have been proposed to explain hemoglobin switching during ontogeny, one assumes a switch in stem cell lineages, each with a different program of globin expression; the other suggests a hemoglobin switch within the same cell lineage that is controlled either intrinsically, and/or may be influenced inductively by cell-environment interactions. The findings that primitive and definitive erythropoietic cells are not clonally determined to make only embryonic or definitive hemoglobin chains, and that fetal and adult chains may be coexpressed in one cell, argue against the first (switch in cell lineage) hypothesis.

The second hypothesis, intralineage switching, has been examined using cell and organ cultures and chick-quail chimeras [55] to evaluate the response of hematopoietic cells of different developmental ages in a developmental environment of a given age. Transplantation models also have been used whereby fetal stem cells are injected into lethally irradiated adult animals and adult hematopoietic cells are injected into a fetus [56,57]. The data demonstrate that changes in the environment may play a role in the hemoglobin switch, but are not the single factor controlling the switch. Switching might reflect a change in the ability of cells to participate in inductive interactions. Other studies suggest that hematopoietic cells have an intrinsic program that directs the switch [58].

Gene structure as well as nuclear-cytoplasmic interactions play a role in the hemoglobin switch. This influence is suggested by the extent to which methylation affects globin expression in man [59,60]. It is also indicated by changes in chromatin organization of globin genes during development, as shown by the genes' sensitivity to DNAse I digestion [61], and by the fact that the genes' expression can be regulated by cytoplasmic proteins (transacting elements), which attach to specific segments of DNA, typically the DNAse I–hypersensitive sites [62].

Studies with transgenic mice have demonstrated that transfected human β globin genes are expressed only in the erythroid cells (tissue-specific expression) and only in cells of the definitive erythroid lineage, not in yolk sac–derived cells (stage-specific expression). Transgenic mice containing human γ globin genes synthesized γ globin only in erythropoietic cells of the yolk sac [63,64]. These studies suggest that only the appropriately staged cells contain the transacting elements that, in addition to *cis*-active DNA segments participate in the control of the hemoglobin switch.

Somatic cell hybrids prepared by fusion of mouse erythroleukemic (MEL) and human cells were also used to study hemoglobin expression. Fetal erythroid cells + MEL cells synthesized fetal γ globin, but with increasing time in culture such cells eventually synthesized the adult program of β hemoglobins. Adult lymphoblasts or fibroblasts + MEL cells synthesized β globin but not γ [65]. The developmental history apparently determines whether the γ gene will be turned on, but the molecular basis for this effect, or for the γ to β switch, is unknown. Nevertheless, the understanding that has already been gained of the developmental regulation of globin genes, and the experimental models used to obtain the data, should be valuable for similar gene regulatory studies of the skin.

Immigrant Cells in the Epidermis of Developing Skin

Melanocytes: Melanocytes are neural crest–derived cells that are thought to enter the epidermis during the first trimester. The timing of this event is based on the observation of cells within the embryonic and early fetal epidermis that possess a nonkeratinocyte nuclear morphology. Other markers of melanocytes, including melanosomes and cell surface antigens, are not present at that time or have not been studied. Melanosomes can be recognized in the cytoplasm of these cells after 60 days EGA, but they do not show morphologic evidence of pigment synthesis and transfer until the second trimester [1].

Whether the neural crest develops into melanocytes or other progeny (glial cells) depends on environmental conditions. Cells destined to become glial cells differentiate into such only if they interact in tissue with neuronal cells; if this interaction is blocked they begin to carry out melanin synthesis. Neural crest cells in vitro also synthesize melanin if they are grown on plastic, but melanogenesis is inhibited if somite-conditioned medium is added to the culture or if cells are grown on a collagen substrate. It is not known how the dermal environment might influence neural crest cells as they migrate into the epidermis and differentiate into melanocytes, or if the dermis affects the pigment cells once they are differentiated. Some interaction between epidermal melanocytes and mast cells of the dermis is, however, implicated in the pathogenesis of neurofibromatosis [66]. The developmental origin of this disorder that manifests itself cutaneously as pigmentation defects or neurofibromas can now be investigated immunocytochemically with the aim of improving our understanding of the ontogenic influences that yield this specific progeny of the neural crest.

Langerhans Cells: Langerhans cells are bone marrow–derived cells of the epidermis [67] that function as antigen-presenting cells to helper T cells to mount an immune response. Like other cells with this function, they bear a number of characteristic antigens (eg, HLA-DR, T200, and S100) and receptors (for Fc-IgG, C3b, Mg^{2+}-activated ATPase) on the cell surface, and express a determinant, OKT6, typically found on immature T cells [68]. They are capable of allogeneic T-cell stimulation in a mixed epidermal cell–lymphocyte reaction and are a source of the cytokine interleukin 1 [69]. Based on the observation of Langerhans cell granules in the cytoplasm, Langerhans cells have been identified in fetal epidermis as early as 10 weeks. More recently, epidermal sheets, prepared from embryonic and fetal skin and examined histochemically and immunohistochemically, have revealed a population of epidermal cells that stain positively for Langerhans cell determinants (ATPase and HLA-DR) in the embryonic tissue and for an additional marker (OKT6) in fetal tissue after approximately 12 weeks [70]. There exist no functional studies of Langerhans cells in vitro nor correlated studies to determine whether

a population of receptive lymphocytes is also present in the dermis at these early ages.

Merkel Cells: Merkel cells in adult skin are located in thick (palmar/plantar) and hairy portions, where they function as slow-adapting mechanoreceptors. They are typically found in assemblies, associated with a single neurite, called touch domes. Dense-core granules that are morphologically similar to monoamine-containing granules are present in the cytoplasm of the Merkel cell, but a specific neurotransmitter has not been identified [71]. Merkel cells may be part of the diffuse neuroendocrine amine precursor uptake and decarboxylation (APUD) system of the body. The origin of Merkel cells is uncertain, although current evidence favors an ectodermal derivation [72]. Like epithelial cells, they contain bundles of keratin filaments and form desmosomes with adjacent keratinocytes. They lack both vimentin and neurofilaments. Merkel cells have not been recognized in fetal skin until late in the second trimester, substantially later than other immigrant cells. This may correlate with their more localized distribution in skin than that of the other immigrant cells, or may be a consequence of not looking at skin from earlier fetuses with the appropriate probes to recognize them.

DERMAL-EPIDERMAL JUNCTION

The dermal-epidermal junction (DEJ) is a basement membrane, separating the epidermis and the dermis, which functions as a zone of interaction, adhesion, and as a semipenetrable barrier between the two tissues. It is involved in a number of hereditary blistering disorders (eg, the epidermolysis bullosa group of diseases) and in certain autoimmune disorders (eg, bullous pemphigoid). Structural regions of the DEJ (lamina lucida, lamina densa) and distinct entities (hemidesmosomes, anchoring filaments, and anchoring fibrils) have been identified, and numerous biochemical constituents (type IV and VII collagens, heparan sulfate proteoglycan, laminin, nidinogen, entactin) and antigens (bullous pemphigoid [BP], KF-1, epidermolysis bullosa acquisita [EBA], and anchoring fibril [AF1 and AF2]) have been recognized in each. The absence of some of the antigenic components (KF-1, AF-1, AF-2) has been correlated with genetic disease.

Ontogenetic studies of the expression of biochemical and antigenic components of the developing DEJ have shown that: (1) type IV collagen is always present in its lamina densa; (2) laminin is expressed in the lamina lucida at all ages, and the BP antigen appears after the epidermis is stratified and hemidesmosomes are structurally complete (around 10 weeks) [73,74]; (3) the EBA antigen is present in the lamina densa of thick skin as early as 8 weeks, when the tissue is stratified [75]; (4) the KF-1 antigen appears in the lamina densa around 16 weeks [73]; and (5) the AF1 and AF2 antigens are expressed after 26 weeks EGA [76], even though structures with the appearance of anchoring fibrils are present in first-trimester skin [1]. There have been no studies to determine whether an antibody to type VII collagen that recognizes anchoring fibrils in adult skin will identify presumptive fetal anchoring fibrils at earlier stages. It is also not known if or how the differentiation of the epidermis and the composition of the dermal matrix at various stages of development determine expression of components of the DEJ. The ontogenetic expression of DEJ structures and antigens has been studied in other development-like systems, such as wound healing, and epiboly in organ culture [73]. Studies to answer these questions in the developing skin could be conducted using culture systems and tissue recombinant methods.

The use of many of the antibodies to DEJ components has been very important in specific diagnosis of genetic diseases involving the DEJ in postnatal skin [76]. Antibodies have recently also been used in studies of frozen sections of fetal skin obtained by fetoscopy. More and improved data underlying this antibody use are, however, needed to allow the continued and expanded use of the antibody procedure for rapid diagnoses of abnormalities in fetal as well as adult skin.

THE DERMIS

Collagen in the Adult and Fetal Dermis Form and function of the dermis are determined by its extracellular matrix, the constituents of which are also found in other connective tissues throughout the body. One of the principal matrix components, collagen, is represented by a family of at least 10 genetically distinct proteins that occur in tissue-specific patterns in the adult and arise during development in a defined temporal sequence. The interstitial collagens (types I, III, and V) form the fibrillar part of the matrix of the dermis. Type VI collagen assembles in beaded filaments adjacent to the collagen fibers; type IV is a component of the basal laminae not only at the DEJ but also around vessels, nerves, and beneath epidermal appendages; and type VII collagen is a component of the anchoring fibrils. The biosynthesis of collagens within the cells of the dermis, including the various enzyme-catalyzed steps, are well understood. The events important in the secretion of collagen molecules into the extracellular matrix and their assembly and stabilization as collagen fibers are also reasonably well understood. Several human collagen genes have been cloned and isolated, as well as partially sequenced, and their chromosomal location determined.

Errors at various levels of collagen biosynthesis have been identified in fibroblasts cultured from patients with inherited connective tissue disorders. Recombinant DNA technology has enabled investigators to define these errors at the gene level [77].

Insight into the developmental regulation of collagen biosynthesis has been obtained through studies of the collagen genes' regulatory mechanisms using cultured fibroblast systems transformed by viruses, or mouse embryos injected with virus. Animal systems, such as *Drosophila* and *Caenorhabditis elegans,* have provided information about the temporal expression of the collagen genes [78].

Using a chick fibroblast system, it was found that cellular transformation with RNA or DNA virus caused a marked reduction in type I collagen synthesis. Injection of a plasmid that contained the promoter region of the cloned chick α 2 type I collagen gene linked to a bacterial gene, into mouse cells (where the plasmid is stably integrated into the genome), followed by viral transformation of the mouse cells, allowed reduction of the synthesis of the bacterial message to the same extent as that of mouse collagen I mRNA. This suggests the same regulation of the collagen promoter after reintroduction into mouse cells as of the endogeneous collagen promoter. Such studies should provide a system for the determination of which promoter sequences may be important for the regulation of collagen genes during development of the skin.

The type of collagen synthesized in fetal skin are the same as those in the adult and they are distributed in the same pattern [79]. In fetal skin, however, collagens III and V are slightly more abundant than in the adult [80]. Higher levels of enzymes related to posttranslational modification of collagen I and III have been noted in the fetus, but mechanisms that regulate prenatal collagen gene expression by fetal fibroblasts have not been explored. The interactions of collagens with other matrix molecules, and cells of the dermis and the epidermis in developing skin are also unexplored.

Collagenase Expression in Development Collagenase is involved in the initial stages of collagen degradation associated with restructuring of the matrix in normal tissue maintenance, pathologic conditions involving tissue inflammation (eg, arthritis) and as part of morphogenetic processes such as wound repair and development. A structurally and enzymatically abnormal collagenase molecule is the basis for the severe inherited blistering disorder, recessive dystrophic epidermolysis bullosa [81]. Collagenase is produced by fibroblasts in the dermis, but must be

regulated stringently so that anomalous patterns of collagen breakdown or accumulation do not occur.

Collagenase expression by fibroblasts cultured from human embryonic and fetal skin increases with progressive gestational age. The amount of catalytically active protein is substantially lower (4–10 times) in fetal fibroblasts than that synthesized by adult fibroblasts, although the amount of translatable collagenase mRNA is only 2–3 times less. These data suggest that there is regulation of collagenase synthesis by the fetal cells at the levels of transcription and translation [82]. Fetal and adult fibroblasts showed similar levels of synthesis of collagenase inhibitory protein [83]. High levels of the inhibitor, however, are present in amniotic fluid and are produced by fetal lung fibroblasts. Purification of some fetal collagenases has revealed that they have low specific activities, and thus there may be structural and/or catalytic differences between these enzymes and those of the adult, suggesting the possibility of synthesis of a fetal type of collagenase [82]. It is not clear whether specific populations of fibroblasts in fetal skin produce collagenase or if all have the same potential. In the adult, collagenase production appears to be maximized by cells near the dermal-epidermal junction [84]. This may suggest an interdependence of epidermal and dermal cells in the regulation of collagenase synthesis that should be investigated in adult and fetal skin.

Epidermal cells from fetal and adult rabbit skin, cocultured with dermal fibroblasts, stimulate collagenase synthesis several fold through the production of a cytokine. Production of this collagenase stimulating substance is cell density– and Ca^{2+}-dependent. Fetal epidermal cells are very effective in the production of the cytokine. The state of differentiation of the epidermal cells that produce this material has not been examined rigorously, although the presence of Ca^{2+} suggests that more-differentiated epidermal cells may be involved [85]. Collagenase synthesis in fetal skin may be correlated with invasive and morphogenetic events such as the formation of epidermal appendages and the establishment of nerve-vascular networks. Purification of the effector substance that stimulates collagenase synthesis, and efforts to identify the production of this material or of a similar acting compound from other dermal cells should receive consideration.

Elastin Expression in Development Elastin, the second major matrix component of the dermis, is the electron-lucent portion that accounts for up to 90% of the mature elastin fiber typically present in the deep reticular dermis of adult skin; the remaining protein is that of the microfibril. Microfibrils are readily apparent in less mature elastic networks in the more superficial dermis. The entire elastin network is continuous and important in the elastic recoil of the dermis after deformation.

Elastin appears to be a late gene product in many fetal tissues, including the dermis. In human fetal skin, elastic fibers are not evident by morphologic criteria until the last few weeks of the second trimester [86]. It is not clear whether the soluble elastin precursor, tropoelastin, can be detected biochemically or immunologically in the tissue at earlier stages. Microfibrils are already recognized in embryonic skin, but there is no evidence that these morphologic entities are biochemically the same as the elastin microfibrils. The control of elastin mRNA in development appears to be largely governed by the amount of elastin mRNA transcribed [87]. Elastin cDNA and genomic clones have been obtained, and the location of the elastin gene on human chromosome 2 has been determined [88]. Use of these probes may prove valuable for further studies of the regulation of elastin synthesis in developing tissues.

Glycosaminoglycans, Proteoglycans, and Glycoproteins in Development Proteoglycans form a gel-like milieu that influences the assembly and organization of collagen and elastin fibers in the dermal matrix. They provide rigidity and compressibility to the dermis, and affect, like other matrix molecules, cell differentiation in both the adult and fetal tissue. Proteoglycans are found in the developing dermis as hyaluronic acid–stabilized proteoglycan aggregates with monomeric units that may exhibit heterogeneity in core protein components. The developmental regulation of proteoglycan synthesis needs to be investigated with regard to the many steps that contribute to the concerted assembly of proteoglycan constituent glycosaminoglycans and core proteins, the latter of which could be approached using antibody and cDNA techniques.

Other matrix components involved in various aspects of cell behavior in the dermis are glycoproteins and specific glycosaminoglycans, which affect the cells' attachment to the extracellular matrix, as well as their migration, proliferation, and differentiation. An example for such a role is that of the predominant glycosaminoglycan, hyaluronic acid, in the embryonic dermis [89]. Hyaluronic acid participates in the formation of pericellular coats and substrates for migrating mesenchymal cells that prevent cellular interaction or adhesion prerequisite for dermal differentiation. Decreases in hyaluronic acid levels correlate with an onset or increase in sulfated proteoglycan biosynthesis at a stage when there is also increased accumulation of collagen and elastin fibers, and restriction in cell mobility occurs [90]. The function of hyaluronic acid and the modulation of hyaluronic acid–cell interactions in the developmental regulation of dermal cell behavior need to be investigated for a better understanding of this matrix component's contributions to the morphogenesis and differentiation of the skin.

EPIDERMAL-DERMAL INTERACTIONS

Epidermal-dermal interactions are important in the regulation of epidermal differentiation (keratinization), morphogenesis (appendage formation), pattern formation (epidermal thickness), and in certain activities of the dermis. The last consequence of these interactions is the least well documented.

Tissue interactions are believed to influence gene expression during organogenesis, but the mechanisms are not well understood. The interaction of the epidermis and dermis during development has already been alluded to, e.g., the epidermal cytokine stimulation of dermal fibroblast production of collagenase. Other clear examples of dermal-epidermal interactions have been revealed by heterotypic and heterospecific epidermal-dermal recombination experiments using developing skin. In such studies, it has been shown that the dermis is responsible for determining whether the skin will be glabrous or bear appendages, and what type of appendage will form and in what distribution [91]. The molecular mechanism for this influence on the developing epidermis is not known. However, morphologic studies and immunohistochemical labeling of antigens and matrix components in the dermis have suggested a distribution of dermal constituents that is different at the site of appendage formation (decreased collagen I and III, increased fibronectin and sulfated glycosaminoglycans) than it is beneath the interappendageal epidermis [92]. These observations suggest that these matrix components may be at least part of the message for epidermal morphogenesis. Other components (type IV collagen, laminin, and heparan-sulfate proteoglycan) remain evenly distributed in the dermis throughout development. The mechanism by which the epidermis is apparently able to sense the microheterogeneous texture of its dermal substrate and respond to it with the appropriate appendages, remains to be studied.

The role of the dermis in regulation of epidermal proliferation and keratinization and the pattern and specificity of keratin biosynthesis has been investigated using the chick as an animal model [93]. It was observed that avian epidermal cells synthesize α and β keratins in a scale-specific pattern, but that the regulation of these patterns resides in the dermis. Dermal tissue from each of the various scale types, recombined with the simple epithelium of the chick chorion and grafted onto the chick chorioallantoic membrane, directs the chorionic epithelium to differentiate and to synthesize keratins according to the scale type from which the dermal tissue was obtained. The studies demonstrate the dermal

capability to direct the epidermis' histogenesis and keratinization [94].

Studies of the scaleless chick mutant (*sc/sc*) have demonstrated the role of the epidermis in the epidermal-dermal tissue interactions. Scaleless embryos do not form the epidermal placodes of scales; however, reciprocal epidermal-dermal recombination experiments using normal and scaleless skin have shown that mutant dermis attains its ability to direct epidermal histogenesis through sequential interactions with a normal epidermis [94–97].

These kinds of studies may provide direction for experiments that could be done with human skin to begin to study disorders (e.g., the ectodermal dysplasias [EDs]) that are thought to result from abnormal epithelial-mesenchymal interactions during development. The EDs are a large, genetically and clinically heterogeneous group of disorders characterized by abnormalities of teeth, hair, sweat glands, and nails—all appendages of the epidermis [98]. The kinds of recombinant studies that have been done in the chick and mouse could be done with human fetal skin to begin to understand the conditions in the epidermis and dermis that are present at the time of appendage formation. With such data, it may be possible to hypothesize how and when during development these disorders could be manifest, and what direction one might take to look for the primary gene defect.

The human skin graft to the nude mouse (as whole skin or in tissue recombinant experiments) has been particularly helpful in identifying whether the genetic defect in an inherited disorder of the skin is primary or secondary, and if primary, whether it is expressed in the epidermis or dermis. This technique has been successful, for example, in the demonstration of the gene responsible for lamellar ichthyosis that causes abnormalities in the epidermis [99]. Use of this system, however, is predicted on having appropriate markers to recognize the abnormality in the tissue either by morphologic or biochemical techniques.

CLINICAL RELEVANCE OF UNDERSTANDING FETAL SKIN DEVELOPMENT

Our understanding of the normal events in cutaneous morphogenesis allows us to predict which inherited disorders of the skin might be expressed in utero, and at which prenatal ages they occur. Insight into both the normal and abnormal development is essential for considerations of prenatal diagnosis and, ultimately, in utero therapy. As prenatal diagnosis for these disorders is carried out, we extend our understanding of the natural history of disease into the prenatal period. At present, some of the limitations in being able to make a diagnosis prenatally are the lack of understanding of the disorders at the gene level, the apparent absence of specific biochemical and/or morphologic markers for the diseases in adult skin and, thus, the lack of probes that might be used to recognize the abnormalities in utero.

In spite of these limitations, there has been considerable success in recognizing several of the more severe inherited disorders of the skin in fetuses that are affected. Some of these disorders can be diagnosed on the basis of a known enzymatic defect using amniotic fluid (e.g., X-linked recessive ichthyosis), or cultured amniotic fluid cells (e.g., Refsum's disease) [100]. Others, for which a biochemical defect has not yet been recognized, can be diagnosed in the adult by a structural abnormality in the tissue (eg, bullous congenital ichthyosiform erythroderma). Understanding the structural, biochemical, and antigenic properties of the skin at relevant stages in development provides a basis for predicting whether an abnormality could be expected to be expressed in utero. Successful prenatal diagnosis, based on recognition of structural markers, has been possible for a number of disorders of keratinization, of the dermal-epidermal junction, and of pigment cell abnormalities [95]. Attempts are also being made to search for structural markers in amniotic fluid cells, or for variations in populations of cells in the amniotic fluid, which may be indicative of the disorder. The goal is to be able to use the amniotic fluid and its cells for an accurate diagnosis, thus to eliminate the more invasive and less available procedure of fetoscopy. Ultimately, one would hope to determine the molecular basis for the disorders and to prepare probes that could recognize the abnormal gene in samples of chorionic villi. When this is possible, the timing of prenatal diagnosis will be moved back from 19–21 weeks (fetoscopy) or 14–16 weeks (amniocentesis) to 8–10 weeks (chorionic villi sampling).

CONCLUSIONS AND RECOMMENDATION

The workshop emphasized that the questions we are asking about skin development are not unique to this organ, or to man, but are general problems in cell and molecular biology that pertain to all of development as they are problems of cell-cell interactions, cell-matrix interactions, tissue interactions, and molecular regulation. Three general areas for future research on cutaneous development were recommended: (1) characterization of various events in cutaneous morphogenesis; (2) regulation of morphogenesis by intrinsic and extrinsic controls; and (3) application of the studies on cutaneous morphogenesis to problems of clinical importance. Most of the specific suggestions for research have been incorporated into appropriate sections in the text. To accomplish many of the suggested goals, it will be necessary to develop cell and organ culture systems of fetal skin and tissues. These may then be used for studies of the regulation of gene expression and to define mechanisms by which cells respond to extracellular cues. DNA probes for structural genes of the epidermis and dermis will need to be prepared that can be used both experimentally and clinically, for example in prenatal diagnosis and carrier testing for genetic diseases. Animal models should be identified and used for experimental and developmental studies that are not possible with the human tissue.

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