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NUTRITIONAL REGULATION OF CULTURED ANALOGUES OF HUMAN SKIN

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Abstract

Applications for a valid analogue of human skin include treatment of full-thickness skin wounds, alternatives to animals for safety testing of consumer products, and investigations of skin biology and pathology. In vitro models of cultured skin have been developed from combinations of cultured human keratinocytes and fibroblasts, and biopolymer substrates, but none has yet demonstrated regeneration of functional epidermal barrier. Formation of epidermal barrier in cultured skin depends greatly on the nutritional composition of incubation media to regulate proliferation and differentiation of keratinocytes into corneocytes and barrier lipids of stratum corneum. To simulate wound healing by keratinocytes, culture media should

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promote rapid proliferation early in the incubation period, followed by reduced proliferation and stimulation of synthesis of corneocytes and barrier lipids. Although functional epidermal barrier has not yet been produced in vitro, transplantation of cultured skin to animals and humans has demonstrated that true epidermal barrier can be generated by cultured epithelium. Understanding of the regulatory factors that promote stratum corneum formation has great potential for the generation of true epidermal barrier in vitro. Standardization and validation of analogues of human skin for therapeutic and diagnostic purposes will lead to meaningful advances in public health and safety.

Comparison of Native and Cultured Skin

Uninjured skin provides a wide variety of protective, perceptive, and regulatory functions for the human body. Protective functions result from epidermal barrier in the stratum corneum, pigment distribution at the dermal–epidermal junction, and from immune effector cells of the epidermis (Langerhans cells) and dermis (mast cells, histiocytes). Perception derives from innervation of the skin, primarily in dermis, that detects touch, temperature, and pain. Other essential functions of skin are thermoregulation by vasomotor response, evaporative cooling of perspiration, and piloerection; and excretion of salt and small organic molecules such as urea. All of these functions are integrated to maintain physiological homeostasis of the boundary between the individual and the environment. Furthermore, epidermal homeostasis is actually a dynamic equilibrium between proliferation of basal keratinocytes and desquamation of mature corneocytes, rather than a "static" physiology.

Injury to skin may be caused by acute or chronic exposure to a toxin or pathogen, by nutritional deficiency, or by trauma, that results in penetration or disruption of the stratum corneum of epidermis. Responses to injury include a wide spectrum of cellular and/or humoral mediators that result in erythema, edema, pain, hyperkeratosis, or ulceration of the skin with subsequent repair and resolution of the injury to eliminate any foreign material, and to restore physiological homeostasis. More recently, direct participation of epidermal keratinocytes in mediation of skin response to injury has been shown to include synthesis and release of immunologic cytokines (interleukins 1, 3, 6, 8; granulocyte and macrophage colony-stimulating factors; prostaglandins), parenchymal cell cytokines (transforming growth factors α, β; basic fibroblast growth factor; hepatocyte growth factor), and attachment factors (collagens IV, V, VII; laminin; fibronectin; E-CAM).[1,2]

Toxicity testing of drugs and consumer products has usually been performed by application of subject compounds to depilated skin of rodents according to the method of Draize.[3] Endpoints of animal tests include ordinal scoring of der-

mal irritancy (erythema and edema) and of corrosion (epidermal disruption, fluid loss, bleeding). Draize tests provide data based on integrated physiological response and allow direct evaluation of formulated compounds, but must extrapolate results from animals to humans, and require large numbers of animal subjects. Public desire for reduction of animals for safety testing of consumer products has generated considerable interest in development of in vitro alternatives to animal testing.[4] Introduction of these testing alternatives may be expected after satisfactory validation of models for individual or multiple endpoints has been established.[5]

Cultured analogues of human skin[6–14] have been proposed as alternatives to animals for toxicity testing, as substitutes for skin autograft,[15,16] and as models for dermatologic research.[17] These models consist of a biopolymer substrate populated with dermal fibroblasts, and epithelialized on one surface with epidermal keratinocytes. Prospective advantages of cultured human skin for toxicity testing include, but are not limited to, greater availability, lower cost per test, species homology, greater uniformity of subject material, and determination of cellular and molecular mechanisms of toxic action. However, none of these models has yet reproduced a fully functional stratum corneum that constitutes the most fundamental anatomical element for establishment of a valid analogue for human skin. Anatomical deficiencies of cultured skin may be expected if conditions for culture and uninjured homeostasis are compared. Correction of anatomical deficiencies will require correction of physiological deficiencies by establishment of a truly physiological culture system. An optimized culture system for skin would contain perfusion of medium with no serum, low/no exogenous growth factors, and a full complement of essential nutrients and vitamins, including essential fatty acids and lipid precursors; ambient atmospheric conditions (humidity, CO_2); and other physical factors including electrical currents, mechanical tension, and fluid pressure. As conditions for cultured human skin become truly physiological, skin analogues may be expected to become skin homologues.

Cell and Tissue Culture of Human Epidermal Analogues

As a first approximation, analogues of human skin should include the main parenchymal cells of dermis, the fibroblasts, and of epidermis, the keratinocytes that are distributed on a collagenous matrix. Because fibroblasts proliferate very well in response to fetal bovine serum (FBS), it remains a standard supplement in media for their growth. However, the proliferative stimulus of FBS on fibroblasts has been replaced by combinations of growth factors (EGF, PDGF), hormones (insulin, hydrocortisone), and lipids.[18] In comparison, FBS does not stimulate growth of epidermal keratinocytes, unless it is combined with cholera toxin and fibroblast feeder cells.[19]

As an alternative, the inhibitory activity of FBS on keratinocytes may be overcome with nutrient media that are biochemically defined.[20-22] Defined media can serve to provide nutritional support permissive for expression of native phenotypes of any cultured cells. Media for keratinocyte proliferation are rich in growth-promoting compounds (peptide growth factors, hormones) and energy sources (glucose, pyruvate, glutamine) that permit expression of the hyperproliferative physiology of keratinocytes after injury. Media for epidermal differentiation and homeostasis, however, contain compounds (essential nutrients and fatty acids) that permit very slow cell replication, and steady progression of keratinocytes through epidermal differentiation to become corneocytes embedded in barrier lipids.[23] Permissive conditions for the re-establishment of epidermal barrier in vitro would simulate wound healing in vivo in which keratinocyte hyperproliferation gradually resolves concurrent or subsequent to formation of functional stratum corneum.

Composition of defined media for epidermal analogues must consider several factors, including, but not limited to, chemical stability, qualitative and

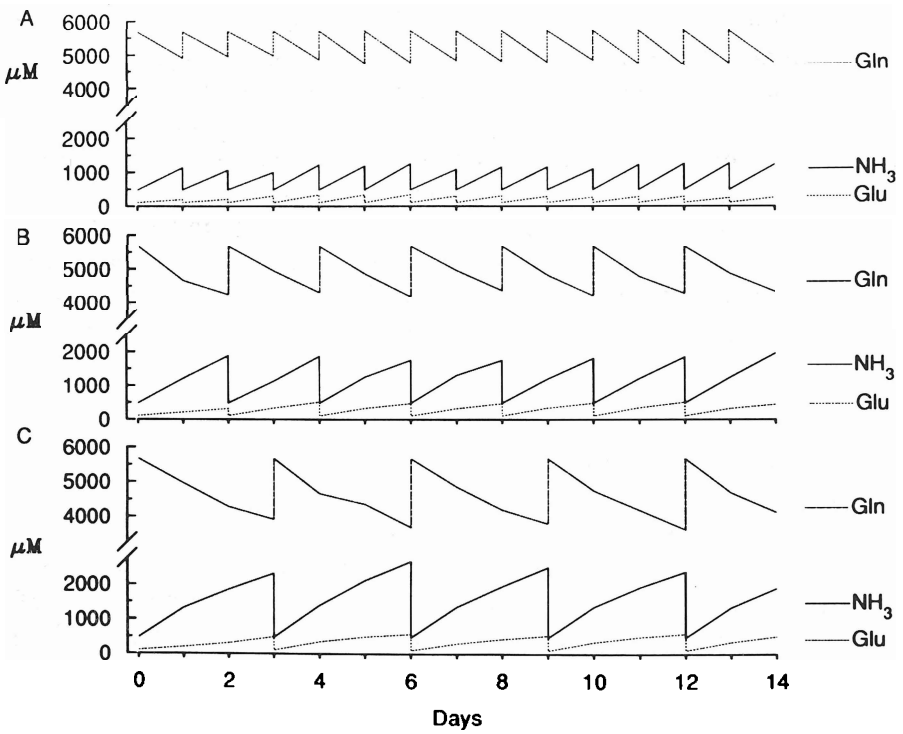

Figure 1. Plot of glutamine (Gln), NH_3, and glutamic acid (Glu) concentrations vs. time of incubation in vitro of cultured analogs of human skin. Media were replaced at intervals of (A) 1 day, (B) 2 days, and (C) 3 days. Analyses of amino acids and ammonia were performed daily.

quantitative levels of basal nutrients, and of regulatory molecules. Figure 1 shows analysis of glutamine (Gln), glutamic acid (Glu), and ammonia (NH_3)[24] plotted against days in culture of composite skin analogues. Media based on MCDB 153 for incubation of cultured skin were replaced at intervals of 1 day (Fig. 1A), 2 days (Fig. 1B), or 3 days (Fig. 1C). These data show an inverse and time-dependent relationship between glutamine and ammonia in a low-protein medium. Enzymatic formation of glutamic acid from glutamine is minimal,[25] although toxic levels of ammonia ($\geqslant 2$ mM) form if media are replaced at 2-day (Fig. 1B) or 3-day (Fig. 1C) intervals. These data suggest that glutamine levels are excessively high in this medium, and that deamination of glutamine produces toxic levels of ammonia. According to the principle of the first limiting factor,[26] toxic levels of ammonia would inhibit cellular responses to all other media components, and may be toxic or lethal to cultured skin analogues.

Another physicochemical parameter is solubility of lipophilic compounds in aqueous media. Examples of this class of compounds are fat-soluble vitamins (retinoic acid, vitamin A; 1,25-dihydrocholicalciferol, vitamin D_3; α-tocopherol, vitamin E), and long-chain fatty acids, including essential fatty acids. Each of these compounds has been demonstrated to have important regulatory activities on epidermal differentiation, but they have not been formulated systematically into serum-free culture media for epidermal analogues. Addition of these compounds requires consideration of effective concentration, which results from both total concentration added and mechanism of delivery.

Essential nutrients also regulate cell-specific function. Figure 2 shows production of one class of epidermal barrier lipids, acylglucosylceramides (AGC),

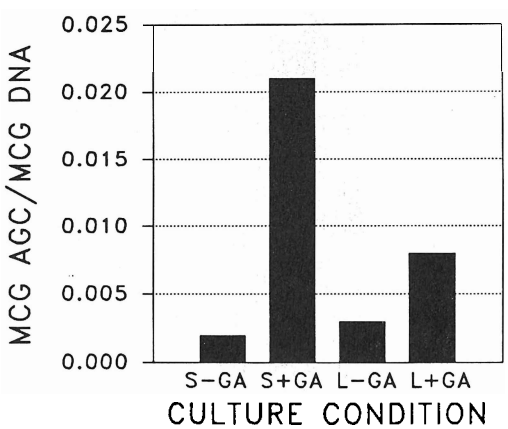

Figure 2. Plot of micrograms (MCG) of acylglucosylceramide (AGC)/microgram of DNA vs. culture condition for cultured analogs of human skin. Culture conditions included saturated (S, 95%) or low (L, 50%) humidity, in media with (+GA) or without (−GA) glucose and acetate.

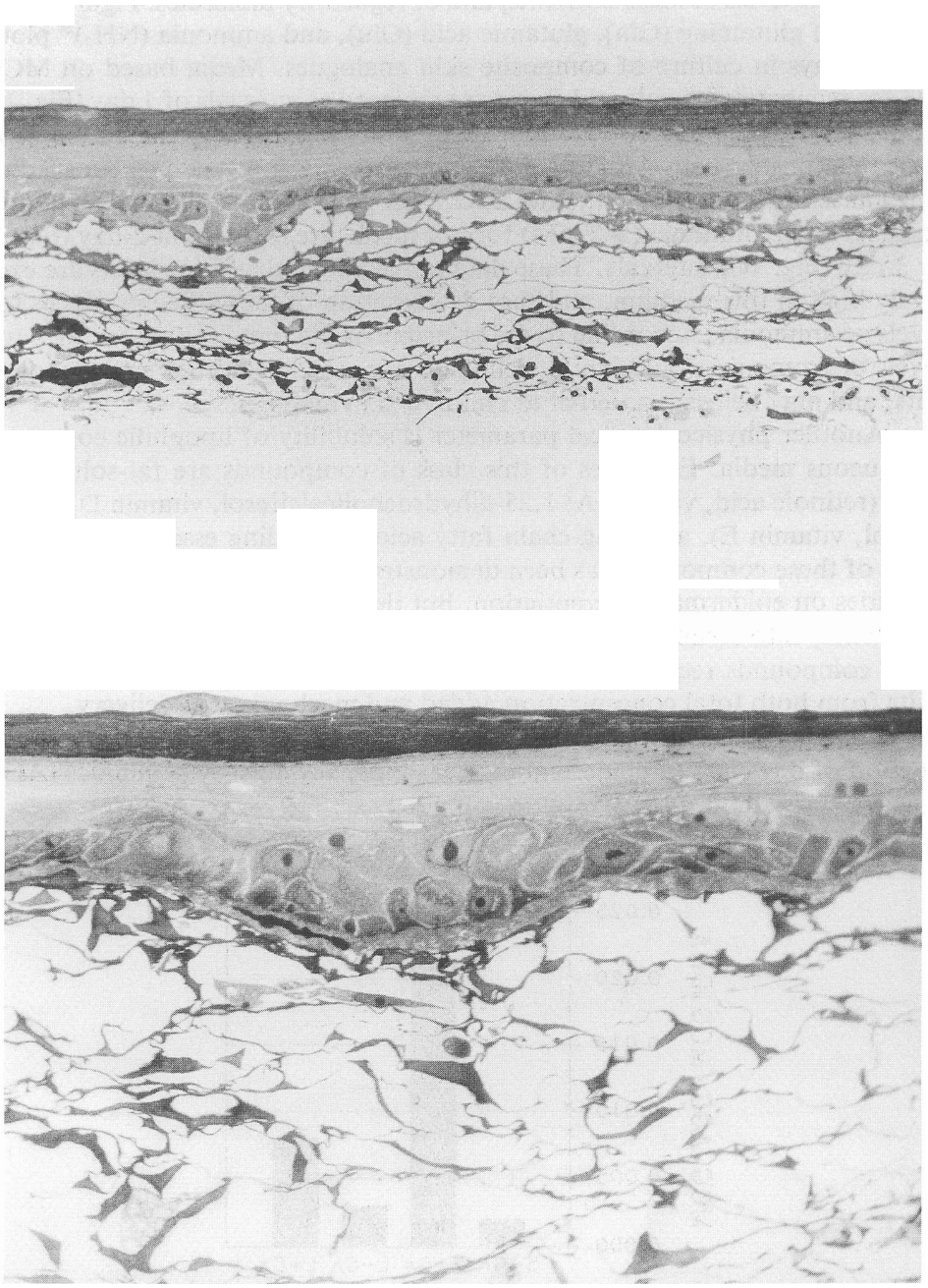

Figure 3. Histologic section of cultured analogue of human skin. a. Analogue consists of collagen-glycosaminoglycan sponge populated internally with cultured human fibroblasts, and on one external surface with cultured human keratinocytes. b. Epidermal component of skin analog contains all epidermal strata.

by cultured skin analogues. In this study, glucose and acetate were included (+GA) or excluded (−GA) from media for cultured epithelium incubated in saturated (95%, "S") or low (50%, "L") humidity. The data suggest that synthesis of barrier-specific lipid, AGC, is glucose dependent, and not influenced significantly by the relative humidity of incubation conditions. However, it is also important to recognize that the highest level of AGC detected in this study is less than 10% of the level in native stratum corneum.

Defects of epidermal barrier in humans are also associated with deficiencies of essential fatty acids,[27,28] linoleic and linolenic acids. As a consequence, many media are supplemented with either linoleic or linolenic acids, but usually not both. However, linoleic (ω-6 unsaturated) and linolenic (ω-3 unsaturated) acids not only contribute to epidermal barrier but also are precursors for prostaglandins of the E_1 and E_2, or E_3 series respectively. Therefore, it may be reasonable to include both essential fatty acids in culture media for skin analogues that do not contain serum. Figure 3 shows the histologic appearance of a skin analogue incubated in low-protein medium containing both linoleic and linolenic acids. Total thickness of the skin analogue is less than 0.3 mm, and the epithelial component contains cell populations that represent the four major strata of native epidermis. Relationships among essential fatty acids in media, stratum corneum structure, and barrier function remain to be investigated.

Figure 4 shows pigmented human epidermis generated from a cultured analogue of human skin with adult human melanocytes.[29] Transplantation to athymic mice restores epidermal barrier[30] and demonstrates that keratinocytes retain the capacity to make barrier.[31,32] Therefore, this model may help to identify factors responsible for the formation of epidermal barrier, and permit formation of functional epidermal barrier in vitro. Advantages of animal model include full physiological function of skin, multiple replicates of the same condition, and control of the genotype of the transplanted cells. Disadvantages include hyperkeratosis and hyperpigmentation of human skin that suggest a latent inflammatory reaction that may be an artifact of the human–murine model. However, transplantation to humans demonstrates that keratinocytes retain the capacity to regenerate true, functional epidermal barrier.[15,16] Further study of transplantation of skin analogues and of mechanisms of skin regeneration may provide crucial insights for the preparation of cultured skin with functional epidermal barrier, and keratinocyte metabolism of native skin.

Current Status and Future Perspectives

Remarkable progress toward preparation of an analogue of human skin has been made in recent years. However, a valid analogue of skin remains an elusive goal primarily due to deficiencies in culture conditions that promote abnormal epidermal physiology. Routine utilization of cultured skin for toxicology will require attainment of three main objectives.

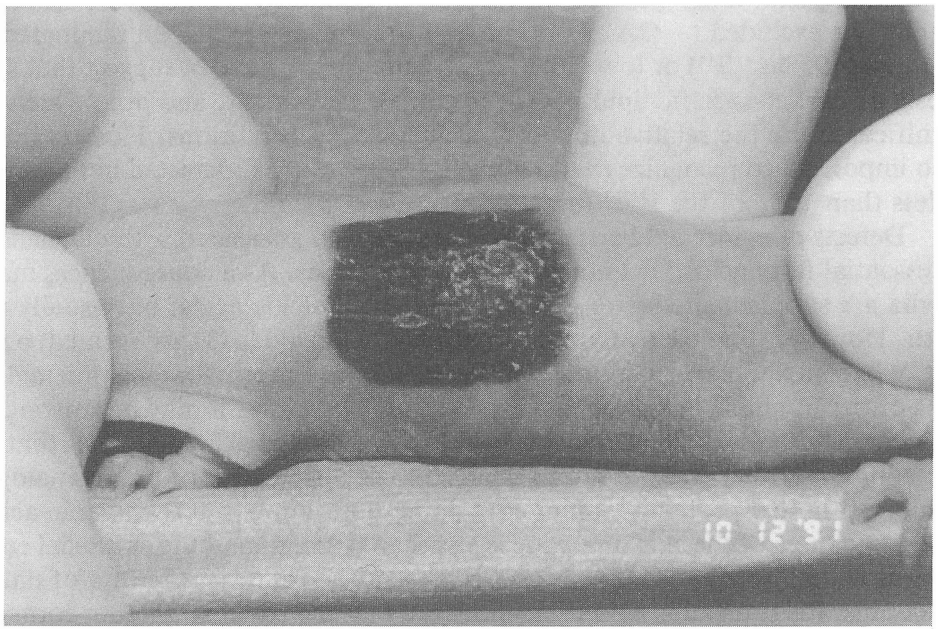

Figure 4. Asthymic mouse with darkly pigmented human skin 7 weeks after receiving a cultured skin graft containing melanocytes.

Improvement of Culture Systems

Deficiencies of culture systems include nutritional, physical, and cellular factors. Nutritional factors for serum-free media include addition of all dietary requirements (e.g., vitamisn A, C, D_3, E; linoleic and linolenic acids), and decrease of exogenous mitogenic compounds (growth factors, hormones) and energy sources after epithelial confluence. Transforming growth factor-α is an autocrine mitogen of keratinocytes at high density. Therefore, exogenous mitogens (i.e., EGF, FGFs) may not be needed after the establishment of stratified epithelium, and are probably not derived from blood in vivo. Decrease of energy sources (glucose, pyruvate, glutamine) may result in greater proportions of barrier lipids (ceramides, AGC), and fewer storage lipids (triglycerides, sterol esters). Physical factors that warrant consideration include reduced humidity,[11,33] perfusion of media to maintain concentrations of metabolites within physiological ranges, and possibly electrical currents or mechanical load. Absence of corneocyte desquamation in vitro is a cellular factor that provides an abnormal influence on equilibrium of proliferation and differentiation in cultured epidermis.

Standardization

After the development of improved culture systems, other factors must be considered to standardize cultured analogues of human skin. Sources of cell strains may introduce variability to skin analogues. Therefore, donor age (neonatal, juvenile, adolescent, adult, geriatric), anatomical site (sun-exposed or non-sun-exposed, palmar or plantar, trunk or limbs), and race require comparative assessment. Also, dynamic changes during the in vitro lifespan of cultured skin dictate limited windows of time for use. At present, no skin analogue reaches true epidermal equilibrium, and none has a culture life greater than 2–4 months. As a consequence, the utility of present models of cultured skin is limited to a finite period of time after "maturity" has been established, but before the analogue has begun to deteriorate. It may be expected that improvements in culture conditions will also contribute to extended in vitro lifespans, and increased periods of practical use.

Validation

Essential to the success of any model of cultured skin is validation in comparison to existing standards for toxicologic testing. Because responses of cultured epithelium are only a subset of in vivo inflammation or corrosion, correlation with animal studies is the best available method for validation. In vitro models with cultured keratinocytes have demonstrated greater than 90% correlation with animal data for dermal irritancy.[34] Although high levels of correlation have been demonstrated, tolerance for false-negative results from in vitro models is extremely low. Therefore, in the interest of public safety, correlation of toxicologic testing with cultured skin versus animals must approach unity. Continual redesign and retesting of models are required to meet this high degree of stringency for test results. Model validation has been described as a multistep process[35] starting with testing by the development laboratory, followed by outside testing and accumulation of consensus data. Satisfactory completion of validation of cultured analogues of human skin for safety testing of consumer products would represent a major advance in public health.

Acknowledgments

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