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Systemic distribution of apolipoprotein E secreted by grafts of epidermal keratinocytes: Implications for epidermal function and gene therapy

(reverse cholesterol transport/secreted protein)

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ABSTRACT **In the present study, human apolipoprotein E (apoE) was monitored in the circulation of athymic mice and rats bearing human epidermal grafts. Human apoE was detected in the systemic circulation of graft-bearing animals as long as the graft remained on the animal. Within 24 hr of graft removal, human apoE was not detectable in plasma, indicating that apoE resulted from continuous production of the protein by grafted keratinocytes. These results show that proteins as large as apoE (299 amino acids) traverse the epidermal–dermal barrier and achieve systemic distribution where they may produce effects on distal tissues. The feasibility of using grafts of genetically-altered keratinocytes for the delivery of secreted proteins is clearly reinforced by the demonstration that an epidermally derived protein exhibits systemic distribution. Finally, by virtue of its systemic distribution, apoE produced in a peripheral tissue such as skin, may function in the reverse transport of cholesterol from peripheral tissues to the liver.**

The primary role of mammalian epidermis is the formation of an impermeable barrier between the animal and the external environment. Recent work has shown that the epidermis is involved in a variety of metabolic activities and may have significant functions beyond a relatively passive role as a barrier (for a review, see ref. 1). Keratinocytes, the principal cell type of the epidermis, synthesize and secrete a variety of cytokines, leading to the suggestion that epidermally derived proteins affect distant cells and organs (2). We previously reported that cultured keratinocytes synthesize and secrete apolipoprotein E (apoE), a major protein component of several classes of mammalian lipoproteins (3, 4). A principal role of apoE in lipoprotein metabolism is as a recognition signal for the hepatic removal of cholesterol-laden HDL subfractions and chylomicron remnants from the circulation (5). In this fashion, apoE serves as a component of the reverse cholesterol transport system, which delivers excess cholesterol from peripheral tissues to the liver for metabolism and elimination (6).

An important question about keratinocyte apoE as well as other keratinocyte secretory proteins is whether such proteins actually reach the systemic circulation. Since the epidermis is not vascularized, secreted apoE must traverse the basement membrane separating epidermis and dermis in order to reach the circulation. In the present study, we have monitored the appearance of human apoE in the circulation of athymic mice that received grafts of cultured human epidermal keratinocytes. As an alternate approach, split-thickness human skin was grafted as a sandwich flap onto athymic rats. In both cases human apoE was detected in the systemic circulation, and production of keratinocyte-derived apoE persisted for as long as 12 weeks. These results establish that a protein secreted by epidermal keratinocytes can reach the systemic circulation. These results also have important implications for the role of keratinocyte apoE in reverse cholesterol transport and for the use of keratinocyte grafts to deliver protein products in somatic gene therapy.

MATERIALS AND METHODS

Cell Culture. Normal human keratinocytes obtained from discarded abdominal, leg, and buttock skin samples were grown to confluency in 150-cm^2 culture flasks on mitomycin C-inactivated 3T3 cells (7) in medium previously described (8). Cultures were fed every 3–4 days, and 24 hr prior to transplantation, were placed in cholera toxin-free and serum-free medium.

Animal Grafts. *Athymic mice.* For cultured grafts, epithelial sheets were detached intact from the surface of the flask by incubation for 1 hr with dispase II (2.5 mg/ml) (9). The detached epithelium was washed once with serum-free, cholera toxin-free medium, and a sterile sheet of Adaptic non-adhering dressing (Johnson & Johnson) was placed on top of the epithelium as supportive backing. Animals were kept under sterile conditions and anesthetized with Metafane gas prior to each procedure. The epithelial sheet on Adaptic backing was grafted basal side down onto a 2 × 1 cm graft bed excised to fascia on the backs of athymic mice. Mock-grafted mice were excised in an identical fashion, and Adaptic moistened with medium was placed on their backs. Grafts were kept in place with 1-inch (2.54 cm) Kling bandages (Johnson & Johnson) and 1-inch Band-Aids. Dressings were removed between 7 and 10 days after grafting. A different human skin donor was used for each experiment.

Athymic rats. Human split-thickness skin grafts (0.5 mm) were generated from routinely discarded skin from elective abdominoplastic surgery and either used immediately or stored in RPMI 1640 medium (GIBCO) with 10% bovine serum at 4°C for up to 72 hr (10). Grafts were placed on the subcutaneous surface of the epigastric skin on the abdomen of the host rat that is supplied and drained by the superficial epigastric artery and vein, respectively (11). The dermis of the donor skin and the subcutaneous tissue of the host skin grow together into a flap sandwiching the vessels supplying the graft. Cyclosporin (2.5 mg/kg of body weight every other day) was administered subcutaneously to prevent rejection of

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Abbreviations: apoE, apolipoprotein E; PAS, periodic acid/Schiff's reagent.

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the human skin graft. Cyclosporin therapy was discontinued 10–14 days prior to the experiments.

Histology. Grafts were excised, fixed in paraformaldehyde, and embedded in paraffin blocks. Sections were examined for basement membrane formation by standard periodic acid/Schiff's reagent (PAS) staining (12). Immunocytochemical analysis using human-specific anti-involucrin antiserum was done according to the manufacturer's instructions (involucrin immunokit, Biomedical Technologies, Stoughton, MA).

Metabolic Labeling. Freshly excised human skin (120 mg) was rinsed in warm phosphate-buffered saline, cut into 2-mm fragments, and incubated at 37°C in 600 μl of methionine-free, serum-free, MCDB (Clonetics) medium containing [^{35}S]methionine (800 Ci/mmol; 1 mCi/ml; 1 Ci = 37 GBq) for 12 hr (13). Medium was harvested, adjusted to contain 0.2 mg of phenylmethylsulfonyl fluoride per ml, centrifuged twice at 12,000 rpm for 5 min. and dialyzed overnight at 4°C against phosphate-buffered saline containing 1 mM methionine.

Immunoprecipitation and Electrophoresis. Aliquots of medium containing 8.5 × 10^5 cpm of trichloroacetic acid-precipitable protein were analyzed for human apoE by immunoprecipitation by the previously described double-antibody procedure (14). Samples were immunoprecipitated with excess rabbit anti-human apoE as the primary antibody and with goat anti-rabbit IgG (Cappell Biochemicals) as the secondary antibody. Immunoprecipitates were analyzed electrophoretically on a 10% sodium dodecyl sulfate (SDS)/polyacrylamide gel (13, 15). As a control, the same sample was treated with preimmune serum and goat anti-rabbit IgG. Purified human plasma apoE was radiolabeled with [^{14}C]-formaldehyde (16) and run in an adjacent gel lane.

Enzyme-Linked Immunosorbent Assay. Human apoE in the plasma of graft-bearing mice and rats was quantified by enzyme-linked immunosorbent assay. Whole blood was immediately adjusted to contain 0.08 mg of phenylmethylsulfonyl fluoride per ml, 10 kallikrein inhibitory units of aproteinin per ml, 0.1% EDTA, 0.05% dithionitrobenzoic acid, and 0.001% Garamycin (gentamycin) and was centrifuged at 10,000 × *g* for 15 min at 4°C to remove blood cells. Plasma samples were stored at −70°C. Enzyme-linked immunosorbent assays were carried out by using a modification of the method of Voller *et al.* (17) as previously described (4). The lower limit of detection of apoE was 0.35 ng/ml.

RESULTS

Persistence of Human Keratinocytes and Reestablishment of Basement Membrane in the Graft Areas. Epithelial sheets of cultured keratinocytes were grafted basal side down onto a 2 × 1 cm excised surface on the backs of athymic mice. Graft survival was typically 50%, with most failures due to poor adherence to the underlying host tissue. At various times

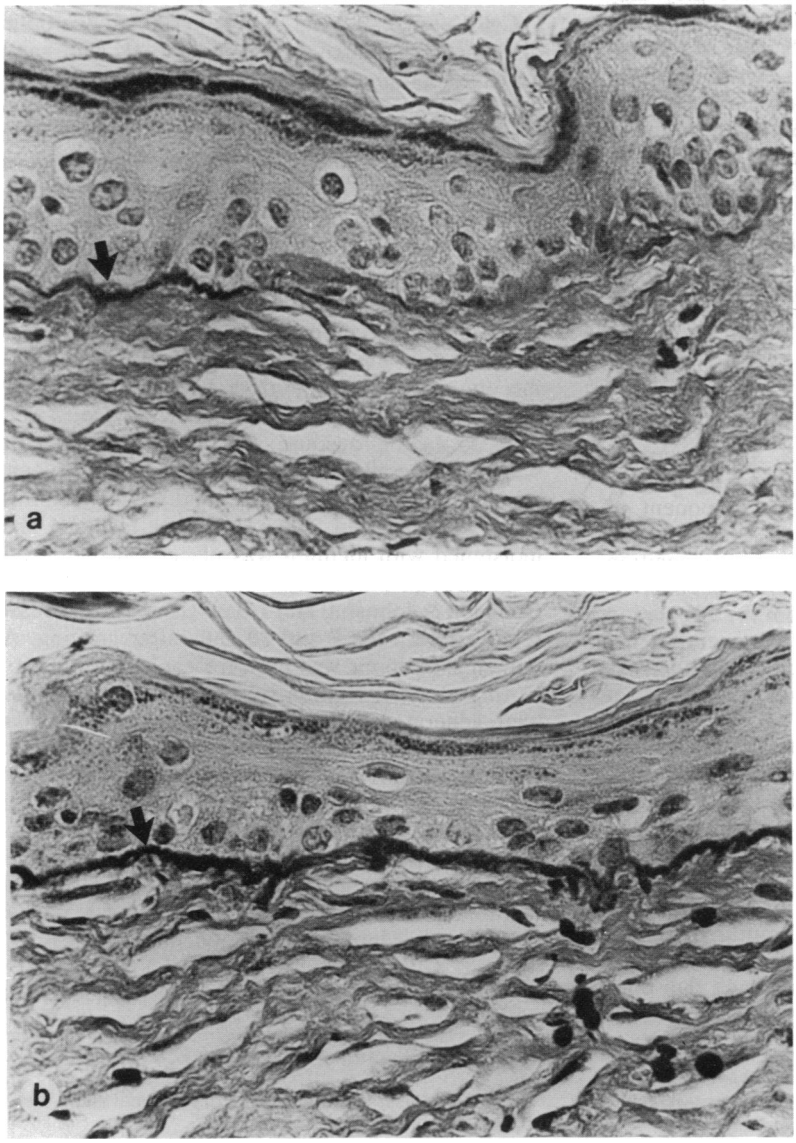

FIG. 1. Morphology of keratinocyte grafts and basement membrane formation. Grafts of cultured human keratinocytes placed on athymic mice were biopsied and stained with PAS. In the cross section of the paraffin-embedded graft, the basement membrane is PAS-positive material and is indicated by the arrow. (*a*) Representative graft excised 7 days after grafting. The discontinuous PAS-positive staining denotes the partially formed basement membrane. (*b*) Representative graft excised 30 days after grafting. The continuous PAS-positive staining indicates a fully formed basement membrane. A stratified squamous keratinizing epithelium is present in these grafts and in all animals that had circulating human apoE.

after grafting, histological analyses were carried out to assess the persistence of human keratinocytes in the surviving grafts (18). Grafts were considered successful when human keratinocytes were present as judged by immunostaining with antibody specific for human involucrin (data not shown). The percentage of total graft area positive for human involucrin varied substantially among grafts.

Basement membrane formation in the graft area was assessed by PAS staining. Substantial reestablishment of basement membrane was noted as early as 7 days after grafting as patchy staining at the epithelial–connective tissue interface (Fig. 1*a*). By 14 days, PAS staining was more uniform, and by 30 days it was continuous (Fig. 1*b*). This result agrees with other studies, which have demonstrated that by 14 days after grafting of human cultured keratinocytes, the dermoepidermal junction was linear, and basal membrane zone antigen, laminin, and type IV collagen were present (19).

Accumulation of Human apoE in Graft-Bearing Animals. Sera from graft-bearing mice were tested for human apoE by using an enzyme-linked immunosorbent assay (4). This assay uses a mouse monoclonal antibody to human apoE and does not detect mouse or rat apoE. Human apoE was detected in mouse serum as early as 4 days after grafting and was present throughout the 46-day observation period (Table 1). The maximum level of apoE detected in an experimental animal was 44 ng/ml. Substantial variation in human apoE concentrations was observed among the animals. These differences likely reflect variation in the number of keratinocytes surviving the graft procedure as well as different rates of apoE secretion among keratinocyte donors (4). Sham-grafted mice were assayed in identical fashion and demonstrated no measurable human apoE above the limit of detection (0.35 ng/ml).

The presence of human apoE in mouse serum for as long as 6.5 weeks (Table 1) suggested that apoE was produced continuously by grafted keratinocytes and was not due to persistence of human apoE released from keratinocytes at or soon after the time of grafting. To confirm this point, grafts were removed from littermates grafted at the same time. Human apoE was not detectable 24 hr after graft removal (Table 1, experiment 4). These results confirm that human keratinocytes persist in the graft and indicate that these cells continue to produce apoE for many weeks.

Further confirmation of the epithelial synthesis and secretion of apoE was investigated by using the human–rat skin sandwich-flap model. In this model system, split-thickness human skin (0.5 mm) is grafted onto a congenitally athymic rat in a skin flap to produce viable human skin with an intact epidermis and some underlying dermal tissue. The grafted tissue is nourished by a single artery and drained by a single vein, the latter of which can be sampled locally prior to entry of this blood into the systemic circulation (11). Ten to 12 weeks after grafting, the concentrations of human apoE in venous drainage from the skin flap were 13.3 and 13.8 ng/ml in two rats grafted at different times. Human apoE concentrations in the contralateral leg vein of these animals were 8.6 and 8.9 ng/ml. The higher concentrations of apoE in the venous drainage from the human skin sandwich flap is indicative of continuous epidermal production of apoE from the grafted skin. These results confirm that apoE production persists in grafted human skin and demonstrate that apoE can reach the systemic circulation from epidermal tissue that is similar in architecture to its normal state.

Table 1. Levels of human apoE in sera of graft-bearing mice

Exp.	Days after grafting	apoE, ng/ml
1	4	15.3
	17	18.9
2	14	32.4
	28	34.5
3	7	21.0
	12	18.0
	21	36.9
	46	30.3
4	9	10.5
	12	7.0
	14	44.0
	14 GR	0.0
	15	13.0
	15 GR	0.0

Enzyme-linked immunosorbent assays were carried out as described. A different skin donor was used in each of the four experiments. In experiment 4, grafts were removed from one of each set of littermates (GR), and 24 hr later serum was tested for apoE. Human apoE was not detectable within 24 hr of graft excision.

Secretion of Newly Synthesized apoE by Full-Thickness Skin. Although cultured and grafted keratinocytes secrete apoE, it was also important to show that apoE synthesis is a property of intact skin and is independent of procedures used for keratinocyte culture or grafting. For this purpose, freshly excised, full-thickness skin was incubated with [^{35}S]methionine for 12 hr in short-term organ culture (13). Secreted proteins were analyzed by immunoprecipitation followed by SDS/10% polyacrylamide gel electrophoresis of the immunoprecipitate. Fig. 2 shows that the anti-apoE antiserum precipitated newly synthesized apoE (lane 1), which comigrated with purified plasma apoE (lane 3). The preimmune serum control did not show the apoE band (lane 2).

DISCUSSION

The results of this study show that apoE produced by human epidermal keratinocytes reached the systemic circulation when keratinocyte grafts were placed on athymic mice. ApoE was present in the circulation prior to and subsequent to reestablishment of the basement membrane between the grafted human cells and host cells. ApoE was also detected in the systemic circulation and in venous blood draining the human skin sandwich flap on athymic rats. These grafts contain an intact epidermis, basement membrane, and dermis at the time apoE was measured (L.K., unpublished observation). These results indicate that basement membrane is not a barrier to the movement of apoE produced by epidermal

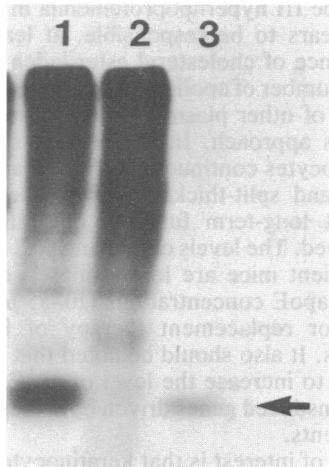

FIG. 2. Secretion of newly synthesized apoE by full-thickness human skin. Immunoprecipitation and SDS/10% polyacrylamide gel electrophoresis were carried out as described. The fluorograph of the gel is seen. Lanes: 1, medium precipitated with excess rabbit anti-human apoE as the primary antibody; 2, the same sample immunoprecipitated with preimmune rabbit serum as the primary antibody; 3, human [^{14}C]apoE.

cells to the underlying cutaneous vasculature in the dermis. These results further demonstrate that expression of the apoE gene, as it occurs in normal human skin, is maintained during keratinocyte culture and persists after grafting. That grafted keratinocytes are capable of continuous production of apoE over prolonged periods of time is of interest in four respects.

First, while it has long been appreciated that epidermally derived, lipid-soluble compounds such as vitamin D can reach the systemic circulation and produce effects on cells in other tissues (20), it was not known whether this was true for epidermally derived proteins. Recent studies show that the cultured keratinocyte is an active secretory cell producing a wide variety of proteins including lymphokines, hormones, growth factors, and apoE (1). The present results indicate that proteins as large as apoE (299 amino acids) traverse the epidermal–dermal barrier and achieve systemic distribution. These findings raise the possibility that other proteins secreted by keratinocytes also achieve systemic distribution and produce effects in distal tissues.

Second, there is considerable interest in the use of skin cells as potential targets for somatic gene therapy. Epidermal keratinocytes and dermal fibroblasts can be grown in culture and grafted back to the host after transfection or transduction with vectors carrying foreign genes (21, 22). In the case of fibroblasts, a "dermal equivalent" consisting of fibroblasts embedded in a matrix of collagen can be placed in subcutaneous tissue (23). Human coagulation factor IX derived from such dermal grafts was detected in the lungs of recipient mice, indicating that a protein derived from a dermal graft could achieve systemic distribution (21). In similar studies, stable transfer of the human growth hormone gene into keratinocytes was achieved with retroviral vectors, and the genetically altered cells were implanted in the subcutaneous tissue of athymic mice (22). In this case, however, human growth hormone was not detected in the serum of graft-bearing animals, although growth hormone was present in the grafts. The failure to detect systemic distribution in the previous study may reflect the site of graft placement or the particular properties of human growth hormone.

Extensive experience with skin grafts in human medicine and the successful use of keratinocyte grafts (24) makes the keratinocyte an attractive cell for delivering a protein product that functions extracellularly or can be targeted to a specific tissue or cell type. This approach might be applicable, for example, in type III hyperlipoproteinemia in which a defective apoE appears to be responsible, at least in part, for reduced clearance of cholesteryl ester-laden remnant particles (5, 25). A number of apolipoprotein deficiency states (26) or deficiencies of other plasma proteins also might be candidates for this approach. In the present study, cultured, grafted keratinocytes continued to produce apoE for as long as 6.5 weeks and split-thickness grafts for 10–12 weeks, suggesting that long-term function of grafted epithelium might be achieved. The levels of human apoE observed in the serum of recipient mice are low (Table 1) compared with normal human apoE concentrations (20–35 μg/ml) but may be adequate for replacement therapy of low-abundance plasma proteins. It also should be noted that little effort has yet been made to increase the level of apoE expression by using stably transduced genes driven by strong promoter and enhancer elements.

A third point of interest is that keratinocyte grafts may be useful in animal studies designed to examine the function, metabolism, and distribution of genetically altered plasma proteins. This approach could be used to examine structure–function relationships *in vivo* or to modulate the concentration of a plasma constituent such as a peptide hormone by use of an inducible promoter. The use of a steroid hormone-responsive promoter to drive expression of a target gene, for example, would permit regulation via topical application of a hormone. The keratinocyte graft approach may provide an alternative to transgenic animals for studies of this nature. Keratinocytes have been cultured from athymic mice (27), BALB/c mice (28), and rabbit (29), and there is no *a priori* reason that they could not be transduced and used as homografts on appropriate hosts.

A final point concerns the functional role of keratinocyte apoE in lipid metabolism. In contrast to most plasma apolipoproteins which are synthesized exclusively in liver and small intestine, apoE is synthesized and secreted by a wide variety of peripheral tissues (14, 30). We have proposed that apoE made in peripheral tissues may function in two aspects of cholesterol and/or phospholipid transport (14). The first is the process of reverse cholesterol transport in which excess cholesterol is removed from peripheral tissues and delivered to the liver for metabolism and elimination (6). The results of this study show formally that keratinocyte-derived apoE does, indeed, reach the systemic circulation as would be required for its participation in reverse cholesterol transport. It should be possible in future studies to determine the distribution of keratinocyte-derived apoE within plasma lipoproteins and to test whether this apoE is subject to removal by the liver.

The second potential role for apoE made in peripheral tissues is in the local shuttling or redistribution of cholesterol among cells within an organ or closed compartment (14). On the basis of experiments with cultured keratinocytes, we previously suggested that keratinocyte apoE may participate in local lipid transport associated with the formation and maintenance of the lipid-rich lamellar layer (4). This layer is located in the intercellular spaces of the upper stratum granulosum and corneum and is a major component of the permeability barrier of the skin (31). The finding that freshly excised human skin synthesized and secreted apoE indicates that apoE is normally made by intact skin and could play a role in these processes.

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