Synthesis and Secretion of Apolipoprotein E by Cultured Human Keratinocytes

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Non-polar lipids are synthesized by keratinocytes in the epidermis and transported to the extracellular space where they contribute to formation of a permeability barrier. Transport of non-polar lipids in other organs and tissues usually occurs with the lipid complexed to an apolipoprotein. In this study we set out to learn if apolipoprotein E is produced by human epidermal keratinocytes in culture.

Analysis of total cellular RNA from cultured keratinocytes showed the presence of human apolipoprotein E mRNA at concentrations ranging from 2.5 to 35 molecules/cell. The cells secrete a protein identified as apo E on the basis of molecular weight, isoform pattern, and immunoreactivity. Enzyme linked immunosorbent assay of media from keratinocyte cultures indicated that apolipoprotein E is secreted at a rate of 0.92 ng/h/10^6 cells. *J Invest Dermatol 92:96–99, 1989*

A primary function of epidermis is to provide a barrier against water loss [1]. It is clear from recent studies that differentiating keratinocytes of the stratum granulosum and stratum corneum mediate barrier formation via production and transport of non-polar lipids from the cytoplasm to the intercellular space [2–4]. In most cases, non-polar lipids do not exit cells without participation of apolipoproteins acting either as carriers or extracellular acceptors [5]. As a group, plasma apolipoproteins are characterized by repeating sequences of amino acids which form amphipathic helices that have one hydrophobic face and one hydrophilic face [6]. This structural feature allows apolipoproteins to associate with and transport nonpolar lipids in an aqueous environment. Most apolipoproteins are synthesized in liver or intestine [5]. Apo E is also synthesized in a wide variety of extra-hepatic tissues (hereafter referred to as peripheral tissues). For example, in human and non-human primates apo E is synthesized in brain, kidney, adrenal gland, spleen, lung, ovary, testis, lymph node, and skeletal muscle [7–9]. In non-human primates apo E mRNA is present in a wide variety of tissues including skin [10].

In light of the role of apolipoproteins in the transport of nonpolar lipids, it is possible that these proteins play a part in epidermal barrier formation. To examine this possibility, we have investigated apo E synthesis and secretion in cultured keratinocytes. In the current study we report that apo E is produced by primary human epidermal keratinocytes in culture.

MATERIALS AND METHODS

Cell Culture Skin samples were obtained from human adult abdomen or newborn foreskin. Epidermal keratinocytes were cultured using techniques [11] and media [12] as described. Mitomycin C-inactivated (Sigma) 3T3 cells were utilized as feeder layers [13,14].

Human hepatocarcinoma cells, HepG2 [15], were cultured in DMEM containing 10% fetal bovine serum (Hyclone Laboratories), 5 μg/ml bovine insulin (Sigma), 100 U/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma).

Metabolic Labeling For metabolic labeling experiments, 10^5 keratinocytes from primary cultures were plated into 35 mm dishes containing 10^5 feeder cells. When cultures reached approximately 90% confluency, cell layers were rinsed twice with warm PBS, and incubated for 30 min at 37°C in methionine-free DMEM. The cultures were then labeled for 4 h in 1.5 ml of methionine-free DMEM containing 200 μCi/ml [^{35}S-methionine (1,200 Ci/mmol, New England Nuclear). Media were harvested, brought to 0.2 mg/ml PMSF, centrifuged twice at 12,000 rpm for 5 min, and dialyzed overnight at 4°C against PBS containing 1 mM methionine.

Immunoprecipitation and Electrophoretic Analysis Aliquots of culture media containing equal amounts of TCA-precipitable protein radioactivity were immunoprecipitated by the previously described double antibody procedure [7]. Immunoprecipitates were analyzed by 10% SDS-PAGE as described by Laemmli [16]. The gels were run at 55 volts for 17 h. After processing for fluorography [17], gels were exposed to Kodak X-Omat AR film for 48-72 h at −70°C. Purified human plasma apo E was radiolabeled

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Abbreviations:

Apo E: Apolipoprotein E
CNS: Central Nervous System
DMEM: Dulbecco Modified Eagle's Medium
ELISA: Enzyme Linked Immunosorbent Assay
IEF: Isoelectric Focusing
IgG: Immunoglobulin G
kb: kilobases
PAGE: Polyacrylamide Gel Electrophoresis
PBS: Phosphate Buffered Saline
PMSF: Phenylmethanylsulfonyl Fluoride
SDS: Sodium Dodecylsulfate
TCA: Trichloroacetic Acid

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with [14]C-formaldehyde [18] and used along with molecular weight markers (Biorad) to calibrate the gels.

Two-Dimensional Gel Electrophoresis High resolution two-dimensional gel analysis was carried out as described by O'Farrel [19]. Samples of culture media from [35]S-methionine labeled cells were immunoprecipitated as above and the resulting pellets were dissolved in 0.05 M N-cyclohexylamine ethanesulfonic acid, pH 9.5; 2% SDS; 5% 2-mercaptoethanol; and 10% glycerol. The samples were electrofocused in parallel tubes for a total of 8,000 Volt-h through ampholines (LKB) mixed as follows: 20% pH 3.5–10.0 and 80% pH 5.0–7.0. For the second dimension, the focused gels were run side-by-side, horizontally, on an 11% SDS-PAGE slab gel to allow direct comparison of the results. Gels were run and processed as described above.

RNA Isolation and Analysis Total cellular RNA was isolated by a variation of the guanidine isothiocyanate method [20].

Aliquots containing 25 μg of total cellular RNA were treated with 6% formaldehyde and electrophoresed through a 1.2% agarose gel as described [20]. The gels were processed and RNA electroblotted to nylon membranes (Genescreen, New England Nuclear) at 500 mAmps overnight under the conditions recommended by the manufacturer. RNA was crosslinked to the nylon membranes by exposure to 254 nm ultraviolet light as described [21]. Prehybridization and subsequent hybridization were carried out as described [22] except the SDS concentration was increased to 5%. The blot was probed with 10^6 cpm/ml nick translated [23] plasmid pE368 (kindly provided by Dr. J. Breslow, Rockefeller University [24]). Blots were washed twice with 2X SSC, 0.5% SDS, for 1 h each at 42°C, followed by twice with 0.1X SSC, 0.5% SDS, for 1 h each at 60°C. Autoradiography of the blots was carried out for 5 d at −70°C.

Absolute concentrations of apo E mRNA were measured by a DNA excess solution hybridization assay of 25 μg aliquots of total cellular RNA. The assay was carried out exactly as described [10].

Enzyme Linked Immunosorbent Assay (ELISA) Rates of accumulation of apo E in media from HepG2 and keratinocytes cultures were measured by ELISA. Cultures were incubated for the indicated times in the appropriate media minus serum. Media were harvested, adjusted to 0.2 mg/ml PMSF, and centrifuged twice at 12,000 rpm for 5 min at 4°C to remove any cells. If necessary, media were concentrated tenfold by lyophilizing to dryness and reconstituting with 1/10 volume of sterile distilled water. Processed culture media were stored at −70°C until used.

ELISA assays were carried out according to a modification of the procedure of Voller et al [25]. Buffer conditions are as follows: buffer I (BI), 0.1 M NaCarbonate, pH 9.6, 0.02% NaAzide; buffer II (BII), 0.9% NaCl, 0.05% Tween 20 (Sigma); buffer III (BIII), PBS, 0.05% Tween 20, 0.02% NaAzide; buffer IV (BIV), 0.05 M NaCarbonate, pH 9.8, 1 mM $MgCl_2$. Assays were carried out in a series of five steps in 96-well EIA plates (NUNC). Between each step the plates were washed 3 times with BII. The entire procedure was carried out at room temperature in a final volume of 0.2 ml/well. The steps were as follows: 1) anti-human apo E monoclonal antibody D3 (supplied by Dr. Eugin Koren, Oklahoma Medical Research Foundation, Oklahoma City) was diluted 1 : 250 in BI and bound to the plate overnight; 2) unknowns, blanks, and dilutions of standardized human sera were added for 2 h; 3) the IgG fraction of a rabbit anti-human apoE polyclonal antiserum (C5; [7]) was diluted 1 : 500 with BIII and added for 1 h; 4) sheep anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) at a 1 : 500 dilution in BIII was added for 1 h; and 5) p-nitrophenyl phosphate (Sigma) at 1 mg/ml in BIV was added until the highest point on the standard curve reaches an optical density (405 nm) of 2.0. Plates were read in a Perkin-Elmer Lambda microtiter plate reader. The lower limit of detection was 0.7 ng/ml in the final assay volume.

RESULTS

RNA Analyses To determine if keratinocytes in culture are capable of producing apo E protein, RNA extracted from various keratinocyte cultures was assayed for the presence of apo E mRNA. Keratinocyte cultures were derived from two samples of newborn foreskin (HFK770 and HFK771) and one sample of adult abdominal skin (HAK010). All three showed a 1.2-kb fragment [26] that hybridized to an apo E cDNA probe. An identical band was seen in the RNA from HepG2 cells (Fig 1).

The cellular concentration of apo E mRNA was determined by DNA-excess solution hybridization assay and found to range from 2.5 to 35 molecules/cell (Table I). These values were substantially lower than seen in HepG2 cells, which contain approximately 726 copies of apo E mRNA/cell.

Protein Analysis Apo E synthesis and secretion in cultured keratinocytes were examined by metabolic labeling with [35]S-methionine and analyzing the media for labeled apo E by immunoprecipitation. As shown in Fig 2, the anti-apo E antiserum precipitated a band from the culture media of abdominal keratinocytes, HAK010 (lane 1), and foreskin keratinocytes, HFK771 *(lane 3)* that co-migrated with both purified human plasma apo E and apo E immunoprecipitated from HepG2 culture media. This band was not immunoprecipitated by pre-immune rabbit serum *(lanes 2,4,6, and 8)* and was not derived from the 3T3 feeder cells because 3T3 cells alone did not produce detectable quantities *(lane 7)*. We conclude that apo E is synthesized and secreted by keratinocytes in culture. As expected from the RNA analysis, the amount of apo E produced by these cells was considerably less than HepG2 cells.

To determine if keratinocytes are capable of post-translationally processing apo E, a two-dimensional gel analysis was carried out. Figure 3 shows that the isoform pattern of keratinocyte apo E is very

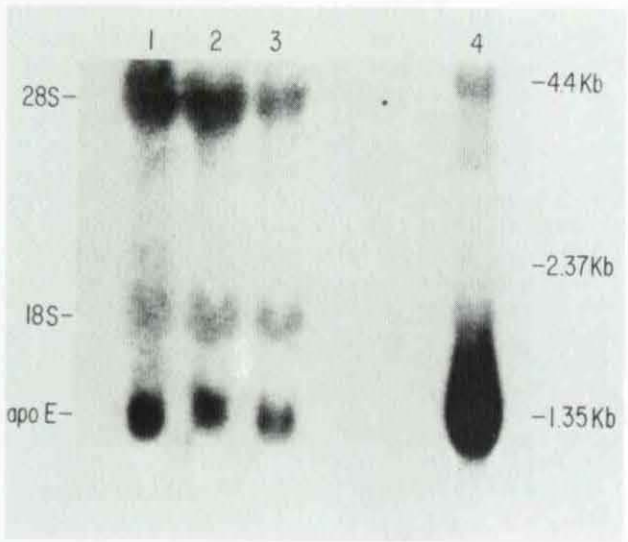

Figure 1. Detection of Apo E mRNA in Cultured Keratinocytes and HepG2 Cells. Total cellular RNA was extracted from cultured keratinocytes and HepG2 cells and analyzed by northern hybridization. The blots were calibrated by RNA molecular weight markers (Bethesda Research Laboratories, Inc.). *Lanes 1,2,3,*and *4* are HAK010, HFK771, HFK770, and HepG2 cells, respectively.

Table I. DNA Excess Solution Hybridization Assay

Sample	Apo mRNA Concentration[a] (molecules/cell)
HFK770	2.5 +/− 0.13[b]
HFK771	9.9 +/− 0.25
HAK010	35.2 +/− 0.28
HepG2	726 +/− 75.6

[a] Calculation of molecules/cell is based on 22 pg total RNA per cell.
[b] Data are expressed as the mean of duplicate determinations +/− standard deviation.

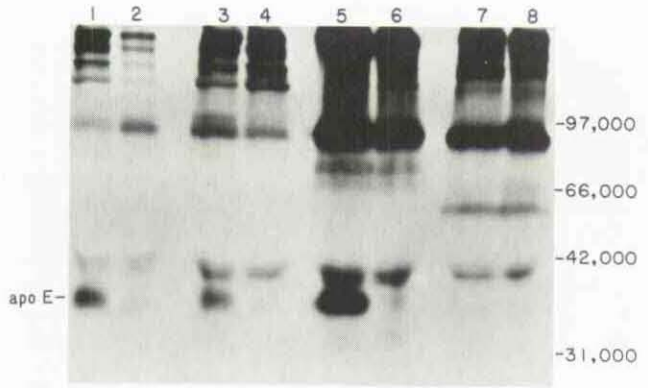

Figure 2. Secretion of Apolipoprotein E From Cultured Keratinocytes. Immunoprecipitation and SDS-PAGE were carried out as described. *Odd numbered lanes* contain samples immunoprecipitated with excess rabbit anti-human apo E as the primary antibody (C5, [7]). *Even numbered lanes* are controls and contain samples immunoprecipitated with pre-immune rabbit serum as the primary antibody. Lane assignments are as follows: *1 & 2,* HAK010; *3 & 4,* HFK771; *5 & 6,* HepG2; *7 & 8,* 3T3.

similar to that of HepG2 cells. The slight differences are due to well-characterized allelic variations and heterogeneity of post-translational sialylation [5,27]. In addition, because keratinocytes produce less apo E than HepG2 cells, less was immunoprecipitated, leaving some isoforms below the level of detection of the procedure. In any case, it is clear that keratinocytes have the capacity to post-translationally process apo E in the proper manner.

Rate of Apolipoprotein E Secretion from Cultured Keratinocytes Rates of apo E accumulation in the media of cultured keratinocytes were determined by ELISA assay. After 12 h in serum-free medium, 1.5 and 60 ng/ml had accumulated in HAK010 and HepG2 cultures, respectively. After 24 h, apo E concentrations had increased to 5 and 110 ng/ml, respectively. After normalization for cell numbers and incubation time, the average rates of accumulation were found to be 0.92 ng/h/10^6 cells for HAK010 and 4.7 ng/h/10^6 cells for HepG2. Rates of protein accumulation in cell culture media are equivalent to rates of secretion only if there is no simultaneous proteolysis. Proteolysis is unlikely because apo E labeled under similar conditions showed no evidence of degradation (Fig 2).

It is interesting to note that while HepG2 cells contain twenty-fold more apo E mRNA than HAK010 cells, they secrete the protein at a rate only 5 times greater. The molecular basis for this difference is not understood.

DISCUSSION

The major finding of this study is that human keratinocytes in culture synthesize and secrete apo E. The data offered in evidence are that keratinocytes make apo E mRNA and a protein, which is identical to apo E by molecular weight, charge, and immunoreactivity. The rate of apo E production as determined by ELISA assay was 0.92 ng/h/10^6 cells. While we offer no direct evidence, we suggest that keratinocytes in epidermis also produce apo E.

Figure 3. Two-Dimensional Gel Analysis. Isoelectric focusing (IEF) was run in parallel tube gels under conditions described. One IEF gel each of HepG2 and HFK771 were placed horizontally on the same SDS-PAGE slab gel, which was run as described. Charge orientation was as shown. After processing, the gel was exposed for 4 weeks.

Table II. Rates of Secretion of Apo E from HAK010 and HepG2

Cell Type	Apo E Concentration (ng/ml culture media)	Rate of Secretion (ng/h/10^6 cells)
HAK010		
12 h	1.5 +/− 0.03[a]	0.89
24 h	5.0 +/− 0.09	0.95
HepG2		
12 h	60.0 +/− 1.5	5.6
24 h	110.0 +/− 1.7	3.8

[a] Data are expressed as the mean of replicate cultures +/− standard deviation.

The functional significance of apo E synthesis in peripheral tissues is unknown. Both a systemic [7,10] and local role [9,28] for peripherally synthesized apo E have been proposed.

The systemic role for apo E involves participation in a process known as reverse cholesterol transport. In this case, apo E synthesized in peripheral tissues mediates transport of excess cholesterol from the tissues to the liver via the blood. At the present time an estimate of the potential contribution to total apo E by the epidermis can only be made with data comparing the relative amounts of apo E mRNA in the different organs and tissues. In a non-human primate (Cercopithecus aethiops, African Green Monkey), the liver contains 60% of total body apo E mRNA, while the adrenals, testis, kidneys, and skeletal muscle contain 0.8%, 2.4%, 14.0%, and 16.0%, respectively. Skin apo E accounts for 1.0% of body apo E mRNA [10]. Epidermis, therefore, likely makes a relatively small contribution to total systemic apo E levels.

A putative local role for peripherally synthesized apo E involves lipid transport within a tissue or closed compartment. For instance, it has been shown that apo E or apo E mRNA is made in rat brain and optic nerve [28] and in astroglial cells of baboon brain [8]. Because apolipoproteins do not cross the blood-brain barrier, any lipid transport role for apo E would have to be local, within the CNS. Specifically, it has been hypothesized that apo E may be involved in cholesterol transport in association with myelin generation and turnover [28].

A local role for epidermal apo E may involve development and maintenance of the lipid rich "lamellar layer" located in the intercellular spaces of the upper stratum granulosum and stratum corneum [2–4]. The lamellar layer contains stacked bilayers that run parallel to adjacent cell membranes [29]. Apo E may participate in the stabilization of this structure. The reason for making this suggestion is that phospholipids that stabilize classical membranes are not present in the lamellar layer. Instead, the lamellar layer is composed of relatively non-polar lipids (ceramides, cholesterol, cholesteryl esters, free fatty acids, and triglycerides) which are unlikely to form a highly structured stacked bilayer [3,30]. It has been suggested that an amphipathic glycoprotein(s) may play a role in stabilization of these bilayer structures [30]. An excellent candidate for such a protein is apo E. The helices of this protein are amphipathic in nature and would allow interaction with both the nonpolar lipid components of the lamellar layer and the aqueous environment, thereby stabilizing the bilayered structures.

Apo E may also be involved in lamellar layer formation in a non-structural capacity. Clearly, the cells of the stratum granulosum move non-polar lipids from the cytoplasm to the extracellular space. In most cases, non-polar lipids exit cells in association with apolipoproteins or rapidly associate with extracellular apolipoprotein acceptors. Perhaps keratinocyte apo E facilitates the transport of permeability barrier lipids from the cells of the stratum granulosum to the site of lamellar layer formation. Further experimentation to determine whether apo E is present in the region of the lamellar layer will be required to clarify the role of epidermally produced apo E.

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REFERENCES

1. Scheuplein RJ, Blank IH: The permeability of the skin. Physiol Rev 51:702–747, 1971
2. Elias PM: Lipids and the epidermal permeability barrier. Arch Dermatol Res 270:95–117, 1981
3. Wertz PW, Swartzendruber DC, Abraham W, Madison KC, Downing DT: Essential fatty acids and epidermal integrity. Arch Dermatol 123:1381–1384, 1987
4. Madison KC, Swartzendruber DC, Wertz PW, Downing DT: Lamellar granule extrusion and stratum corneum intercellular lamellae in murine keratinocyte cultures. J Invest Dermatol 90:110–116, 1988
5. Mahley RW, Innerarity TL, Rall SC, Weisgraber KH: Plasma lipoproteins: apolipoprotein structure and function. J Lipid Res 25:1277–1294, 1984
6. Brewer HB: Current concepts of the molecular structure and metabolism of human apolipoproteins and lipoproteins. Klin Wochenschr 59:1023–1035, 1981
7. Blue M-L, Williams DL, Zucker S, Ali Khan S, Blum CB: Apolipoprotein E synthesis in human kidney, adrenal gland, and liver. Proc Natl Acad Sci 80:283–287, 1983
8. Lin C-T, Xu Y, Wu J-Y, Chan L: Immunoreactive apolipoprotein E is a widely distributed cellular protein; immunohistochemical localization of apolipoprotein E in baboon tissues. J Clin Invest 78:947–958, 1986
9. Williams DL, Dawson PA, Newman TC, Rudel LL: Apolipoprotein E synthesis in peripheral tissues of nonhuman primates. J Biol Chem 260:2444–2451, 1985
10. Newman TC, Dawson PA, Rudel LL, Williams DL: Quantitation of apolipoprotein E mRNA in the liver and peripheral tissues of nonhuman primates. J Biol Chem 260:2452–2457, 1985
11. Rheinwald JG, Green H: Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6:317–330, 1975
12. Wu Y-J, Parker LM, Binder NE, Beckett MA, Sinand JH, Griffiths CT, Rheinwald JG: The mesothelial keratins: a new family of cytoskeletal proteins identified in cultured mesothelial cells and nonkeratinizing epithelia. Cell 31:693–793, 1982
13. Rheinwald JG: Serial cultivation of normal human epidermal keratinocytes. Meth Cell Biol 21:229–254, 1980
14. Macpherson I, Bryden A: Mitomycin C treated cells as feeders. Exp Cell Res 69:240–241, 1971
15. Aden DP, Fogel A, Plotkin J, Damjanov I, Knowles BB: Controlled synthesis of HB_5Ag in a differentiated human liver carcinoma-derived cell line. Nature 282:615–616, 1979
16. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685, 1970
17. Bonner WM, Caskey RA: A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur J Biochem 46:83–88, 1974
18. Rice RH, Means GE: Radioactive labeling of proteins in vitro. J Biol Chem 246:831–832, 1971
19. O'Farrell PH: High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021, 1975
20. Maniatis TE, Fritsch EF, Sambrook J: Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982
21. Church GM, Gilbert W: Genomic sequencing. Proc Natl Acad Sci 81:1991–1995, 1984
22. Thomas PS: Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc Natl Acad Sci 77:5201–5205, 1980
23. Rigby PWJ, Diekmann M, Rhodes C, Berg P: Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J Mol Biol 113:237, 1977
24. Zannis VI, McPherson J, Goldberger G, Karathanasis SK, Breslow J: Synthesis, intracellular processing, and signal peptide of human apolipoprotein E. J Biol Chem 259:5495–5499, 1984
25. Voller A, Bidwell D, Bartlett A: Molecular enzyme immunoassays for the immunodiagnosis of viral infections. In: Rose N and Friedman H (eds.): Manual of Clinical Immunology. American Society of Microbiology, Washington D.C., 1976, p 506
26. McLean JW, Elshourbagy NA, Chang DJ, Mahley RW, Taylor JM: Human apolipoprotein E mRNA. J Biol Chem 259:6498–6504, 1984
27. Zannis VI, Breslow JL: Human very low density lipoprotein apolipoprotein E isoprotein polymorphism is explained by genetic variation and post-translational modification. Biochemistry 20:1033–1041, 1981
28. Dawson PA, Schechter N, Williams DL: Induction of rat E and chicken AI apolipoproteins and mRNAs during optic nerve degeneration. J Biol Chem 261:5681–5684, 1986
29. Elias PM: Epidermal lipids, membranes, and keratinization. Int J Dermatol 20:1–19, 1981
30. Lampe MA, Burlingame AL, Whitney J, Williams ML, Brown BE, Roitman E, Elias PM: Human stratum corneum lipids: characterization and regional variations. J Lipid Res 24:120–130, 1983