Lipoxygenase mRNA in cultured human epidermal and oral keratinocytes

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Abstract Although 15-lipoxygenase has not been purified from cultured human keratinocytes nor has cDNA coding for the protein been isolated from this source, this enzyme activity is implied by the finding of its stereospecific product in in vitro experiments. Based on two primer pairs derived from human reticulocyte 15-lipoxygenase cDNA, we detected ~260 bp and ~370 bp cDNA fragments that were indistinguishable by gel electrophoresis and Southern hybridization from those derived from reticulocyte 15-lipoxygenase cDNA. The ~260 bp polymerase chain reaction (PCR) fragment from a keratinocyte plasmid cDNA library was cloned and determined, by sequencing, to contain 262 bp that were 100% identical to the corresponding reticulocyte 15-lipoxygenase cDNA. These two reticulocyte-type 15-lipoxygenase PCR fragments were also detected from oral keratinocytes. Using platelet-type 12-lipoxygenase cDNA primers, we derived a 264 bp cDNA fragment from keratinocyte mRNA. By sequence analysis, this fragment was determined to be 99.6% identical to that from platelet-type 12-lipoxygenase cDNA. The same fragment was also observed from two amplified keratinocyte cDNA libraries, and from oral keratinocyte mRNA. This is the first demonstration of reticulocyte-type 15-lipoxygenase cDNA derived from the mRNA of cultured human keratinocytes.—**Zhao, H., B. Richards-Smith, A. N. Baer, and F. A. Green.** Lipoxygenase mRNA in cultured human epidermal and oral keratinocytes. *J. Lipid Res.* 1995. **36:** 2444–2449.

Supplementary key words 12-lipoxygenase • 15-lipoxygenase • keratinocyte differentiation

Lipoxygenases are non-heme iron-containing enzymes that catalyze the dioxygenation of polyenoic fatty acids or their derivatives containing one or more 1,4-*cis,cis*-pentadiene structures (1–3). The 12- and 15-lipoxygenases are closely related both in structure and in gene location (chromosome 17) (4). 15-Lipoxygenase activity has been detected in human reticulocytes (5), airway epithelia (6, 7), eosinophils (4), and keratinocytes (8, 9). The biological roles of 12- and 15-lipoxygenase are not completely established (10). The only well-studied case suggestive of physiological activation of 15-lipoxygenase in vivo has been in the reticulocyte (11). In vivo activation of this enzyme is thought to render mitochondria more susceptible to proteolysis, thereby preparing the reticulocyte for maturation to an erythrocyte (12). There is also strong evidence that the 15-lipoxygenase of keratinocytes is activated in such diseases as psoriasis and other scaling dermatoses (13–15). As a potentially key observation, we have found esterified 15-lipoxygenase products in scales from patients with psoriasis and other scaling dermatoses (13). This finding would suggest that the 15-lipoxygenase activation is not merely an epiphenomenon of terminal differentiation in the corneocyte. Moreover, there is suggestive evidence that some degree of 15-lipoxygenase activation may occur normally in the epidermis (16). There are a number of observations in different cell types pointing to a role of 15-lipoxygenase products in normal differentiation (17–22). Whether such a role also applies to the normal keratinocyte is unknown.

It is not clear whether there is only one 15-lipoxygenase or whether there are several cell-specific enzymes that are closely related (23). Platelet-type 12-lipoxygenase cDNA has recently been identified in freshly isolated human epidermal cell preparations (24, 25). Using a combination of RT–PCR and DNA hybridization, we have for the first time detected reticulocyte-type 15-lipoxygenase and platelet-type 12-lipoxygenase cDNA reverse-transcribed and amplified from the mRNA of three strains of cultured human keratinocytes and three cDNA libraries likewise prepared from cultured keratinocytes.

Abbreviation: RT-PCR, reverse transcriptase–polymerase chain reaction.

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MATERIALS AND METHODS

Cell culture and oral epithelial cells

Keratinocytes were prepared in this laboratory from four newborn foreskins, and were cultured by the classical method of Rheinwald and Green (26). Oral keratinocytes were obtained by gentle scraping of the buccal cavity (27). Papanicolaou smears showed that all of the buccal cells were classified as "intermediate" or "superficial" in the Frost classification by cytology (28).

mRNA preparation

mRNA was isolated from the cultured human keratinocytes and non-cornified keratinocytes of the oral cavity using Invitrogen's FastTrack kit (Invitrogen, San Diego, CA). The cells were washed at 4°C in phosphate-buffered saline and lyzed in the detergent-based buffer. The mRNA was then separated by chromatography on oligo (dT) cellulose and finally precipitated with 2.5 volumes of ethanol. The yield ranged from 3 to 5 μg mRNA per 10^7 cells.

cDNA library construction

Approximately 4 μg of mRNA isolated from each of two keratinocyte strains was used for first strand cDNA synthesis using the ZAP-cDNA synthesis kit (Strategene, La Jolla, CA). The double-stranded cDNA was directionally cloned into *EcoRI-Xho1* sites of the Uni-Zap XR vector arms. The lambda library was packed in Gigapack II Gold packaging extract and plated on the *E. coli* cell line XL1-Blue MRF. The final library consisted of ~1.1 × 10^6 independent colonies with a recombinant frequency of 96.7%. A third keratinocyte cDNA library cloned into a pSPORT1 plasmid was prepared by the method of Tseng and Green (29).

Hybridization

Hybridization and probe labeling were carried out using Gene Images kit and Random Primed Images Biotin labeling kit (US Biochemical, Cleveland, OH), respectively, according to the manufacturer's protocols. PCR products derived from keratinocyte mRNA and from the cDNA libraries were electrophoresed in a 1.2% agarose gel and transferred to a nylon membrane. The membrane was hybridized with a biotin-14-dCTP-labeled cDNA clone, specific for either 15- or 12-lipoxygenase. Reticulocyte-type 15- and platelet-type 12-lipoxygenase cDNA clones were generous gifts from Drs. E. Sigal (Syntex Laboratories, Palo Alto, CA) and C. Funk (Division of Clinical Pharmacology, Vanderbilt University, Nashville, TN), respectively. These two cDNAs were also obtained from Oxford Biomedical Research (Oxford, MI). The membrane was washed in 2 × standard saline citrate (SSC)/0.5% sodium dodecyl sulphate at room temperature and then in 0.2 × SSC/0.1% sodium dodecyl sulphate at 50°C followed by 65°C. After a final wash in 2 × SSC, the membrane was subjected to chemiluminescent detection as recommended by the manufacturer, and then was exposed to Kodak X-OMAT AR X-ray film at room temperature for 10–20 min.

Oligonucleotide primers

Oligonucleotides (column-purified) were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). All the primers were lipoxygenase cDNA-specific and their sequences are shown in **Table 1.** The DNA primers specific for platelet-type 12-lipoxygenase were constructed to contain extensions with either *EcoRI* or *XbaI* restriction sites at their 5′-ends (31). This procedure resulted in the addition of 24 bases to the PCR product.

TABLE 1. Oligonucleotides and PCR product size for 15- and 12-lipoxygenases

cDNA Species	5′ Primers	Corresponding Nucleotides	3′ Primers	Corresponding Nucleotides	Size of PCR Product (bp)	Reference
Reticulocyte 15-Lipoxygenase	CATGAGCTGC AGTCTCATCTT CTGAGGGG	1066–1094	AGAAGGAGCTG TAGGTTAGGA AGGC	1303–1327	262	30
Reticulocyte 15-Lipoxygenase	GGCAAGGAGA CAGAACTCAA GGTG	133–156	TCCAGAAATCG CTCATCCACAG GG	483–506	374	30
Platelet 12-Lipoxygenase	TGGCTCCTGG CAAAGTCCTG	1018–1037	CTGTACATGGC CCCCTCCAC[a]	1262–1281	288	31

[a]For reasons of brevity, the added restriction sites (*XbaI* and *EcoRI*) are not indicated, but are included in the size of the PCR product.

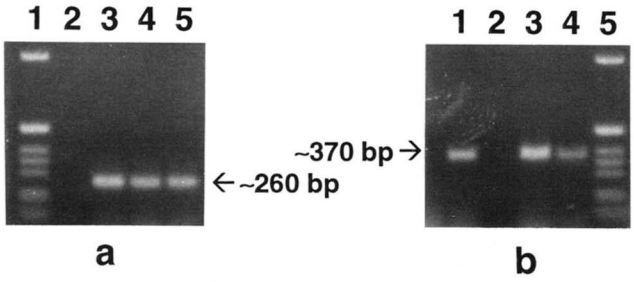

Fig. 1. Two distinct PCR fragments derived from a keratinocyte plasmid cDNA library and from human reticulocyte 15-lipoxygenase cDNA have the same electrophoretic mobility. Gel electrophoresis of the ~260 bp fragments is shown in panel a and that of the ~370 bp fragments is shown in panel b. In panel a, lane 1, 1 kb ladder; lane 2, template-free control; lane 3, reticulocyte 15-lipoxygenase cDNA; lanes 4 and 5, keratinocyte plasmid cDNA library, duplicates. In panel b, lane 1, reticulocyte 15-lipoxygenase cDNA; lane 2, template-free control; lanes 3 and 4, keratinocyte plasmid cDNA library (different concentrations); lane 5, 1 kb ladder.

cDNA synthesis and PCR amplification

Thirty to 50 ng mRNA were reverse-transcribed in 20 µl reverse transcriptase 10 mM Tris-HCl (pH 8.3) buffer containing 90 mM KCl, 1.0 mM $MnCl_2$, 200 µM dNTP, 0.75 µM downstream primer, and 5 units of *Thermus thermophilus* (rTth) DNA polymerase (Perkin-Elmer, Forest City, CA). For PCR amplification, the following components were added to the RT reaction mixture: 8 mM Tris-HCl (pH 8.3), 80 mM KCl, 0.04% Tween 20, 600 µM EGTA, 4% glycerol, 1.5 mM $MgCl_2$, and 0.15 µM upstream primer. PCR was then carried out under the following conditions: initial denaturation at 95°C for 1 min; for each subsequent cycle, denaturation at 95°C for 20 sec; annealing and extension at 60°C for 35 sec, for a total of 40 cycles which is below the 45–50 cycles recommended by the manufacturer.

PCR amplification from each of the plasmid and phage cDNA libraries was carried out in 50 µl PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3) containing 200 µM dNTP, 1.5 mM $MgCl_2$, 0.3 µM of each primer, and 1.25 units of *Thermus aquaticus* DNA polymerase (Perkin-Elmer, Forest City, CA). The reaction conditions were: 95°C, 1 min; 55°C, 1 min; 72°C, 1 min; 30 cycles.

Cloning and sequencing

The PCR fragments were purified from the PCR reaction using ProMega's Wizard PCR preps DNA purification system, and ligated into pGEM-5Zf(+) using pGEM-T vector system II. (ProMega, Madison, WI). Recombinant plasmid was isolated and purified with Wizard minipreps DNA purification system. (ProMega, Madison, WI). The plasmid insert was sequenced by the double-stranded dideoxy chain-termination method (32) using primers T7 and SP6 contained in the pGEM-5Zf(+) vector. Sequencing was performed at the Center for Advanced Molecular Biology and Immunology, the State University of New York at Buffalo.

Controls

Positive controls included authentic human platelet 12- and human reticulocyte 15-lipoxygenase cDNAs, and mRNA similarly prepared from the human epithelial tumor line BT20, which contains 12- and 15-lipoxygenase mRNA (33). Negative controls consisted of template-free incubations and mRNAs prepared from fibroblasts of the same donor as the cultured keratinocytes.

RESULTS

Presence of 15-lipoxygenase cDNA in three keratinocyte cDNA libraries and three keratinocyte strains

Based on the hypothesis that keratinocyte 15-lipoxygenase cDNA contains extensive sequences that are homologous to reticulocyte 15-lipoxygenase, two primer sets derived from sequences of reticulocyte 15-lipoxygenase segments (Table 1) were synthesized and used in PCR with a keratinocyte cDNA library as a template. A ~260 bp fragment and a ~370 bp fragment were produced from plasmid and phage cDNA libraries derived from three strains, and demonstrated by electrophoresis on 1.5% agarose gel. These fragments were indistinguishable in size from those derived from reticulocyte

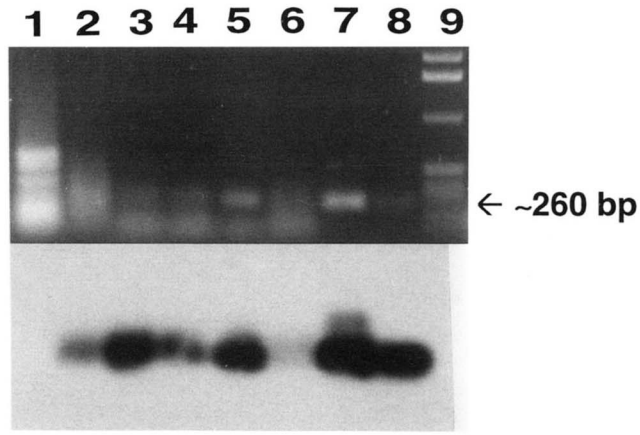

Fig. 2. The PCR fragments (~260 bp) derived from three strains of cultured keratinocytes, oral keratinocytes, and a keratinocyte phage cDNA library are indistinguishable from the fragment derived from reticulocyte 15-lipoxygenase cDNA. Upper panel, gel electrophoresis: lane 1, pBR 322/*Msp*I ladder; lanes 2, 3, and 4, three keratinocyte strains; lane 5, oral keratinocytes; lane 6, BT-20 cell line; lane 7, phage cDNA library; lane 8, reticulocyte 15-lipoxygenase cDNA; lane 9, 1 kb ladder. Lower panel, Southern hybridization of gel in upper panel with a biotinylated reticulocyte 15-lipoxygenase cDNA probe.

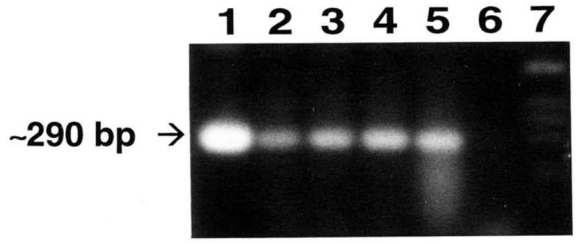

Fig. 3. The ~290 bp PCR fragments derived from two cultured keratinocyte strains are indistinguishable by gel electrophoresis from the fragment derived from platelet 12-lipoxygenase cDNA. Lane 1, platelet 12-lipoxygenase cDNA; lanes 2 and 3, control BT-20 cells (two concentrations); lanes 4 and 5, two keratinocyte strains; lane 6, template-free control; lane 7, 1 kb ladder.

15-lipoxygenase cDNA (**Fig. 1** and **Fig. 2**). Furthermore, Southern blot analysis indicated that the labeled 15-lipoxygenase probes hybridized to both the keratinocyte library-derived ~370 bp and ~260 bp fragments as well as those from the positive controls. The ~260 bp fragment amplified from the phage library is shown in Fig. 2.

The ~260 bp amplified cDNA derived from the keratinocyte plasmid library was purified directly from the PCR reaction, cloned, and sequenced. This cDNA was determined to be 262 bp in length and 100% identical to the corresponding human reticulocyte 15-lipoxygenase cDNA.

RT-PCR was used to directly amplify keratinocyte mRNA utilizing the above-listed reticulocyte 15-lipoxygenase primer sets. One ~260 bp low density band (Fig. 2) and another ~370 bp band (data not shown) were found by electrophoresis, and their identity was confirmed using Southern blot analysis.

Presence of platelet-type 12-lipoxygenase cDNA in three keratinocyte strains and in three cDNA libraries

An intriguing question is why 12-lipoxygenase activity is completely silent in cultured keratinocytes despite the finding of its mRNA and its product by others in epidermal cell preparations (24, 25). Cultured keratinocytes do not contain all the cell types present in normal epidermis but, on the other hand, this source is free of such cells as platelets, whose total absence from epidermal cell preparations is difficult to confirm with certainty. We therefore sought evidence for the presence of the 12-lipoxygenase transcript in cultured human keratinocytes. PCR was used to amplify keratinocyte 12-lipoxygenase mRNA utilizing platelet 12-lipoxygenase cDNA primers (Table 1). The PCR product was ~290 bp, and was indistinguishable in size from the fragment produced with a known platelet 12-lipoxygenase cDNA template (**Fig. 3**). This ~290 bp 12-lipoxygenase fragment was detected from three keratinocyte strains. The identity of the PCR product was verified by hybridization studies using a labeled platelet 12-lipoxygenase cDNA probe (**Fig. 4** and **Fig. 5**).

The ~290 bp PCR fragment amplified from keratinocyte mRNA was purified, cloned, and sequenced. The cDNA fragment was determined to be 264 bp (after exclusion of added restriction sites) in length and showed 99.6% identity to the corresponding human platelet 12-lipoxygenase cDNA. The alteration of the coding sequence at nucleotide 1208 gave glycine for the keratinocyte enzyme, instead of alanine for amino acid 403.

When the keratinocyte plasmid and phage cDNA libraries were used in PCR as templates, the same ~290 bp fragment was generated. A labeled platelet 12-lipoxygenase cDNA probe was found to hybridize to this fragment from the plasmid library (Fig. 4). It should be noted that, as expected, the primers designed for 12-lipoxygenase did not give a product when used against the 15-lipoxygenase cDNA template and vice versa. Fibroblast-derived mRNA was consistently negative (data not shown).

Presence of 15- and 12-lipoxygenase mRNA in non-cornified keratinocytes of the oral cavity

Non-cornified human oral keratinocytes produce 15(S)- and 12(S)-HETE from exogenous substrate without membrane damage, in contrast to cultured cells which produce only 15-lipoxygenase products and only after membrane damage (27).

As in the cultured cells, RT-PCR carried out with the

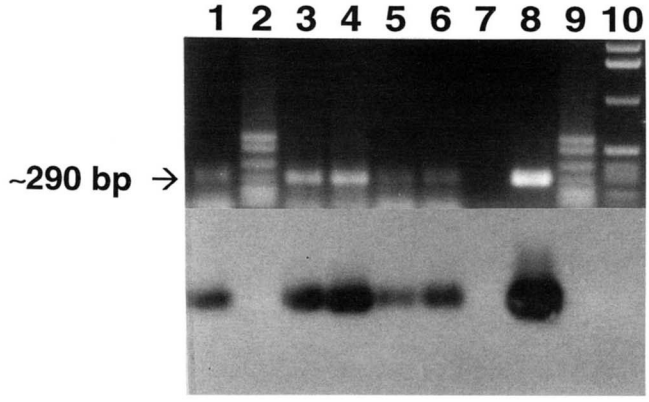

Fig. 4. The ~290 bp PCR fragments derived from two cultured keratinocyte strains and a keratinocyte plasmid cDNA library are indistinguishable from that derived from platelet 12-lipoxygenase cDNA. Upper panel, gel electrophoresis; lane 1, plasmid cDNA library; lanes 2 and 9, pBR 322/M*sp*I ladder; lanes 3 and 4, duplicates of one keratinocyte strain; lanes 5 and 6, duplicates of a second keratinocyte strain; lane 7, template-free control; lane 8, platelet 12-lipoxygenase cDNA; lane 10, 1 kb ladder. Lower panel, Southern hybridization of the gel in the upper panel, using a biotinylated platelet 12-lipoxygenase cDNA probe.

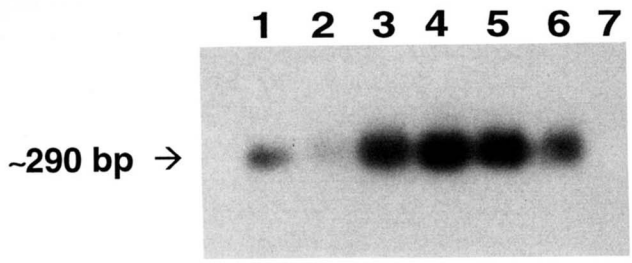

Fig. 5. The ~290 bp fragments derived from each of three keratinocyte strains and from oral keratinocytes are comparable to that derived from platelet 12-lipoxygenase cDNA by Southern hybridization using a biotinylated platelet 12-lipoxygenase probe. Lane 1, platelet 12-lipoxygenase cDNA; lane 2, BT-20 cells; lane 3, oral keratinocytes; lanes 4–6, three different keratinocyte strains; lane 7, template-free control.

two indicated reticulocyte 15-lipoxygenase primer sets and oral keratinocyte mRNA, yielded two fragments of ~260 bp and ~370 bp. These fragments were indistinguishable in the mobility by electrophoresis to those from reticulocyte 15-lipoxygenase. The labeled reticulocyte 15-lipoxygenase cDNA probe hybridized to these fragments. The results showing the ~260 bp fragment are given in Fig. 2.

A ~290 bp PCR product was observed by electrophoresis when oral cell mRNA was reverse transcribed and amplified utilizing the platelet 12-lipoxygenase cDNA primers (Table 1). The PCR fragment was indistinguishable in size to that from platelet 12-lipoxygenase, and the labeled platelet 12-lipoxygenase cDNA probe hybridized to these fragments (Fig. 5).

DISCUSSION

We have documented the occurrence of both platelet-type 12- and reticulocyte-type 15-lipoxygenase mRNA in cultured human keratinocytes using a combined technique of PCR, Southern hybridization, and sequencing. The possibility of illegitimate transcription (34) must always be considered when many cycles of PCR are required to generate a recognizable product. However, in this case, where stereospecific enzymatic activity can be demonstrated, one would have to assume that two different 15- and two different 12-lipoxygenases are present in these cells. The consistent negativity of the fibroblast controls from the same donor as the keratinocytes would argue against this possibility, as all cells should exhibit the same phenomenon (34). Moreover, the positive findings for each enzyme from two libraries cannot result from such transcription.

In vivo activation of the 15-lipoxygenase of human keratinocytes may have important biological and pathological consequences (35). The finding of 13 (predominantly S)-hydroxyoctadecadienoic acid from normal skin surfaces (16) suggests that there may be some degree of activation of 15-lipoxygenase in vivo. In addition, there has been a substantial number of studies of different cell types implicating a role of 15-lipoxygenase products in differentiation (17–22), but such a role has yet to be established for the keratinocyte. The quantitation of 15-lipoxygenase mRNA in keratinocytes that had been separated by size (degree of differentiation, (36)) could be a first step in the elucidation of such a role.

The substitution of guanine for cytosine at nucleotide 1208, giving glycine instead of alanine for amino acid 403, could represent an allelic variant or a PCR amplification error. Given the presence of 12-lipoxygenase mRNA in cultured human keratinocytes, it remains unexplained why these cells do not exhibit 12-lipoxygenase activity by product formation, regardless of the method of stimulation or damage. Whether this absence is an artifact of the submerged cultured state, or whether it is a normal property only of the less differentiated cell, could be answered by studies now under way using air–liquid interface cultures and cells forced to differentiate in viscous media. The fact that oral keratinocytes exhibit both 12- and 15-lipoxygenase activity (27) suggests that the extent of differentiation could be an important factor.

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