Expression of Cloned Human Reticulocyte 15-Lipoxygenase and Immunological Evidence That 15-Lipoxygenases of Different Cell Types Are Related

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Cloned 15-lipoxygenase has been expressed for the first time in eukaryotic and prokaryotic cells. Transfection of osteosarcoma cells with a mammalian expression plasmid containing the cDNA for human reticulocyte 15-lipoxygenase resulted in cell lines that were capable of oxidizing body arachidonic acid and linoleic acid. The lipoxygenase metabolites were identified by reverse-phase and straight-phase high pressure liquid chromatography, ultraviolet spectroscopy, and direct mass spectrometry, verifying that the cDNA for 15-lipoxygenase encodes an enzyme with authentic 15-lipoxygenase activity. Incubation of the transformed cells with arachidonic acid generated 15-hydroxyeicosatetraenoic acid (HETE) and 12-HETE in a ratio of 8.6 to 1, demonstrating that 15-lipoxygenase can also perform 12-lipoxygenation. Lesser amounts of 15-keto-ETE, four isomers of 8,15-diHETE, and one isomer of 14,15-diHETE were observed. Incubation with linoleic acid generated predominantly 13-hydroxy linoleic acid. The reaction was inhibited by eicosatetraenoic acid but not by indomethacin. Antibodies to a peptide corresponding to a unique region of the predicted amino acid sequence were generated and shown to react with one major band of 70 kDa on immunoblots of human leukocyte 15-lipoxygenase. To obtain antibodies to the full length enzyme, the cDNA was subcloned into a bacterial expression vector and was expressed as a fusion with the CheY protein. The overexpressed protein was readily purified from bacteria and was shown to be immunoreactive to the peptide-derived antibody. Antibodies raised to this recombinant enzyme did not cross-react with human reticulocyte 5-lipoxygenase but did identify 15-lipoxygenase in rabbit reticulocytes, human leukocytes, and tracheal epithelial cells, suggesting that the 15-lipoxygenases from these different cell types are structurally related.

The enzyme 15-lipoxygenase catalyzes the insertion of molecular oxygen into arachidonic acid at carbon 15 (1). The enzyme can also oxygenate other polyenoic free fatty acids (2, 3) as well as a variety of phospholipids (4). This ability to perform lipid peroxidation is manifested in multiple biological systems. For example, 15-lipoxygenase appears to contribute to cellular differentiation in the reticulocyte (2), and to the generation of inflammatory mediators in leukocytes (1, 5), and in human airway epithelial cells (6, 7). These potential biological actions for 15-lipoxygenase have led to increasing interest in understanding the molecular mechanism of the enzyme. Soybean 15-lipoxygenase has been studied extensively, and cDNAs for three different isozymes have been reported (8–10). The mammalian form of the enzyme has been purified to homogeneity from rabbit reticulocytes (2) and from human leukocytes (11). Using protein sequence information from the mammalian enzymes, we recently isolated a cDNA encoding 15-lipoxygenase from a human reticulocyte library (12). The nucleotide sequence of this cDNA has allowed us to deduce the amino acid sequence of the enzyme and to identify potential domains critical to enzymatic function based on sequence similarity to other lipoxygenases. The nucleotide sequence from the rabbit reticulocyte lipoxygenase has since been reported (13), and the predicted amino acid sequence is 81% identical to the human enzyme. However, expression of cloned 15-lipoxygenase from any species has not been described. Active expression of the cDNA is critical to establishing authenticity of the isolated clones and to investigating relationships between the enzyme structure and its function.

Investigators of the rabbit reticulocyte 15-lipoxygenase have presented immunological evidence that the enzyme is specific to red cells (2). Whether the 15-lipoxygenase activity detected in various human tissues is due to the same enzyme or isozymes is unknown. Antibodies to human 15-lipoxygenase will be useful in defining these relationships, as well as in studying enzyme regulation and cellular localization. The biological investigation of 15-lipoxygenase in general has been hampered by the lack of antibodies to the human enzyme. This deficiency has been due to the difficulty in obtaining adequate quantities of pure human enzyme. Our prior success in isolating human 15-lipoxygenase was due to the observation that patients treated with interleukin-2 resulted in large quantities of eosinophil-enriched leukocytes (11, 14). Because changes in clinical protocols preclude ready availability of this source, molecular biological techniques were essential to provide reagent levels of the enzyme.

In this paper we describe the active expression of cloned
human reticuocyte 15-lipoxygenase in human osteosarcoma cells. The expressed enzyme exhibits characteristics that are indistinguishable from native 15-lipoxygenase, adding important verification of the authenticity of the 15-lipoxygenase cDNA. Furthermore, antibodies to human 15-lipoxygenase have been generated to a peptide fragment of the deduced amino acid sequence and to the recombinant enzyme expressed in bacteria. Immunoblot analysis with these antibodies demonstrates that the 15-lipoxygenase of leukocytes, airway epithelial cells, and reticuocytes are structurally related.

**MATERIALS AND METHODS**

Purified rabbit reticuocyte lipoxygenase was prepared from anemic rabbits by a modification of the procedure of Rapoport et al. (2) using ammonium sulfate precipitation and hydrophobic-interaction chromatography (11). The purified enzyme generated predominantly 15-hydroxyeicosatetraenoic acid (HETE) from arachidonic acid. Enriched human leukocyte 15-lipoxygenase and homogenous human 5-lipoxygenase were kindly provided by Dr. Carol Rouzer, Merck Frosst, Canada, using chromatographic procedures previously described (15). Both human and mouse leukocytes were activated and generated the arachidonic acid metabolites from arachidonic acid. The expression vector pJC264 was kindly provided by Dr. Jon Condra, Merck, Sharp and Dohme. The human osteosarcoma cell line 143.89.2 was isolated from J. Knutson and R. Suglen, University of Wisconsin Medical School. Airway epithelial cells (>95% purity) were isolated from human trachea obtained less than 24-h post mortem by the method of enzymatic dissociation (16). Arachidonic acid and linoleic acid were obtained from NuChek Prep (Elysian, MN). The ionophore A23187 was kindly provided by Calbiochem. Authentic reference compounds included (15(S)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HETE), (12(S)-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HETE), 15-keto-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-KETE), 8(S),15(S)- and 8(R),15(S)-dihydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (diHETEs), 14(R),15(S)-dihydroxy-5Z,8Z,10E,12E-eicosatetraenoic acid (14,15-diHETE), (13(S)-hydroxy-9Z,11E-octadecadienoic acid (13-HODE), 13-keto-9Z,11E-octadecadienoic acid (13-KODE) from Biomol. (Pymouth Meeting, PA); (R),(15(S)- and (S),(15(S)-dihydroxy-5Z,9Z,11E,13E-eicosatetraenoic acid (8,15-Leukotrienes) were kindly provided by Dr. J. Rakoh, Merck Frosst Canada; prostaglandin B2, 5,8,11,14-eicosatetraenoic acid (ETYA) and indomethacin were from Sigma. Cell culture supplies were from the University Cell Culture Facility, and all reagents were of the highest commercial grade.

**Mammalian Cell Expression**—The vector pR135 was chosen for mammalian cell expression studies. This vector contains a cytomegalovirus immediate early promoter, a polylinker-cloning site, the bacterial selectable marker gene hygromycin B resistance, a eukaryotic origin of replication, and a bovine growth hormone-derived signal sequence (17). The expression vector pJC264 was kindly provided by Dr. Jon Condra, Merck, Sharp and Dohme. The human osteosarcoma cell line 143.89.2 was isolated from J. Knutson and R. Suglen, University of Wisconsin Medical School. Airway epithelial cells (>95% purity) were isolated from human trachea obtained less than 24-h post mortem by the method of enzymatic dissociation (16). Arachidonic acid and linoleic acid were obtained from NuChek Prep (Elysian, MN). The ionophore A23187 was kindly provided by Calbiochem. Authentic reference compounds included (15(S)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HETE), (12(S)-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HETE), 15-keto-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-KETE), 8(S),15(S)- and 8(R),15(S)-dihydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (diHETEs), 14(R),15(S)-dihydroxy-5Z,8Z,10E,12E-eicosatetraenoic acid (14,15-diHETE), (13(S)-hydroxy-9Z,11E-octadecadienoic acid (13-HODE), 13-keto-9Z,11E-octadecadienoic acid (13-KODE) from Biomol. (Pymouth Meeting, PA); (R),(15(S)- and (S),(15(S)-dihydroxy-5Z,9Z,11E,13E-eicosatetraenoic acid (8,15-Leukotrienes) were kindly provided by Dr. J. Rakoh, Merck Frosst Canada; prostaglandin B2, 5,8,11,14-eicosatetraenoic acid (ETYA) and indomethacin were from Sigma. Cell culture supplies were from the University Cell Culture Facility, and all reagents were of the highest commercial grade.

**Plasmid DNAs were transfected into the osteosarcoma cells by calcium-phosphate precipitation (19). Briefly, 10 pg of plasmid DNA was removed from the cells, which were then treated for 2 min with 1.2% acetic acid (v/v), pH 3.5, and one volume of chloroform. An aliquot of prostaglandin B2 was used as an internal standard. All extracts were washed with water and were reconstituted in methanol for storage at -70 °C under N2.**

**High Pressure Liquid Chromatography (HPLC) of 15-Lipoxygenase Products**—Extracts were reconstituted in chromatography solvent and were analyzed by reverse-phase (RP) HPLC on a Waters 840 chromatography system using a Dynamax C-18 column (6 μm, 4.6 mm x 25 cm, Rainin). The column was developed at a flow rate of 1.0 ml/min by a gradient program using two solvents (A and B) set at 50% B for 0-19 min, 70% B for 20-39 min, and 100% B for 40-60 min, where A was methanol/water/acetic acid (50:50:0.1) and B was methanol/water/acetic acid (90:10:0.1). The HPLC eluate was monitored using a Waters 490 multiform length detector set at 210 nm to detect leukotrienes and diHETEs, 235 nm for monoHETEs, and 210 nm for linoleic acid and arachidonic acid. Products were quantitated using standard molar absorption coefficients. Selected compounds were collected from the RP-HPLC eluate, freeze-dried, and analyzed by electron-impact mass spectrometry (VG Analytical, Manchester, United Kingdom). Electron-impact mass spectra were obtained using 70-eV ionizing voltage at 220 °C.

**Ultraviolet Spectroscopy and Mass Spectrometry of 15-Lipoxygenase Products**—To further confirm the identity of the metabolites formed by the recombinant enzyme, selected peaks detected on RP-HPLC were collected from the eluate and were reextracted and dissolved in methanol to determine absorption spectra. The ultraviolet spectra were determined in a diode array spectrophotometer (Hewlett Packard, model 8451A). Metabolites were also characterized by their mass spectra. The compounds were converted to their trimethylsilyl derivatives (6) and were analyzed by electron-impact mass spectrometry on a VG-70SE mass spectrometer using a direct insertion probe (VG Analytical, Manchester, United Kingdom). Electron-impact mass spectra were obtained using 70-eV ionizing voltage at 220 °C.

**Generation of Antibodies**—The 21-amino acid peptide sequence NH2-KKLWPARGLKLYEPEYL-COOH corresponding to the predicted amino acids 37-57 deduced from the cDNA 15LOX (12) was synthesized on an Applied Biosystems model 430 using tert-butyloxycarbonyl amino acids and phenylacetamidomethyl resin. The peptide was conjugated to bovine serum albumin using carbodiimide chemistry (20) and was used to generate rabbit antiserum (Berkeley Antibody Co.). To obtain antibodies to the entire human enzyme, the 2722-base pair EcoRI fragment of 15LOX was subcloned from Bluescript (Stratagene) into the EcoRI site of the bacterial expression vector pJC264. This vector is designed to express heterologous proteins fused with the bacterial signal peptide. When cleaved from the bacterial enzyme, the 2722-base pair EcoRI fragment of 15LOX was used to transform Escherichia coli, and the bacterial proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) for the appearance of recombinant 15-lipoxygenase. Expressed proteins were further studied for immunoreactivity with the peptide-derived antibody. E. coli transformed with the vector containing the insert was grown in the presence or absence of 5,10-RSJC264 (15) (Fig. 1), was used to transform Escherichia coli, and the bacterial proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) for the appearance of recombinant 15-lipoxygenase. Expressed proteins were further studied for immunoreactivity with the peptide-derived antibody. E. coli transformed with the vector containing the insert was grown in the presence or absence of 5,10-RSJC264 (15) (Fig. 1), was used to transform Escherichia coli, and the bacterial proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) for the appearance of recombinant 15-lipoxygenase. Expressed proteins were further studied for immunoreactivity with the peptide-derived antibody. E. coli transformed with the vector containing the insert was grown in the presence or absence of 5,10-RSJC264 (15)
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Protein was readily purified by differential solubility (21) and was used to generate polyclonal antibodies (Berkeley Antibody Co.) which were purified by ammonium sulfate precipitation and affinity chromatography on Sepharose G (Pharmacia LKB Biotechnology Inc.). The antibodies did not reduce the formation of 15-HETE from arachidonic acid by either purified rabbit reticulocyte or enriched human leukocyte 15-lipoxygenase when preincubated with enzyme for 4 h at 4 °C, suggesting that the antibodies are not neutralizing.

Immunoblot Analysis—SDS-PAGE was performed on 10% polyacrylamide gels overlaid with a 3% polyacrylamide stacking gel as described (22). Separated proteins were then transferred to nitrocellulose filters, and immunoblot analysis was performed (23) with either the peptide-derived or the CheY-15-lipoxygenase antibody as a primary antibody. The secondary antibody was goat anti-rabbit IgG linked to horseradish peroxidase (Bio-Rad), and the blots were developed as described (24).

RESULTS

Creation of 15-Lipoxygenase-containing Cell Lines—The cDNA for 15-lipoxygenase was subcloned into the expression vector pR135 for mammalian expression (17) (Fig. 1). This vector contains the hygromycin-selectable marker for isolation of the transformed cells and the cytomegalovirus immediate-early promoter for control of transcriptional expression. The presence of the Epstein-Barr virus origin of DNA replication, “oriP,” permits the plasmid to replicate extrachromosomally in cells containing the Epstein-Barr virus EBNA1 protein (25).

Plasmids with and without the 15-lipoxygenase cDNA were transfected into the EBNA1-positive human osteosarcoma cell line 143.98.2 by the calcium-phosphate method. Individual hygromycin-resistant clones were screened for 15-lipoxygenase activity. Enzyme activity was not detected in the nontransfected cells or in any of the colonies (n = 5) transfected with the vector alone. In contrast, 6 out of 10 clones transfected with the cDNA for 15-lipoxygenase exhibited enzyme activity. The formation of lipoxygenase products varied from clone to clone and ranged from 230 to 4530 pmol of 13-HODE generated/10⁶ cells in 15 min. The variability in expression levels presumably results because the expression plasmid is episomal, and its gene copy number varies more

Fig. 1. Map of vectors used for mammalian and bacterial expression. A, individual components of the plasmid pCMV15LOX used to transfect osteosarcoma cells are shown along with selected restriction sites. B, components of the plasmid pCheY15LOX used to transform E. coli are shown along with selected restriction sites. C, the junction sequence between CheY and 15LOX is shown. The amino acid sequence is denoted using the one-letter code. The vertical bar denotes the beginning of the 5′-untranslated region of 15LOX. The first amino acid in the native 15-lipoxygenase sequence is indicated in bold (M). The EcoRI site is indicated by the arrow.

Fig. 2. RP-HPLC chromatograms of osteosarcoma cells assayed for lipoxygenase activity after transfection with pCMV15LOX or pR135. A, OS15LOX(+) cells (5 x 10⁶) containing 15LOX were incubated with arachidonic acid (160 μM) and the cell supernatants were extracted and analyzed by RP-HPLC as described under “Materials and Methods.” Peaks are labeled to correspond with the compounds identified in Table I. B, OS15LOX(+) cells (1 x 10⁷) were incubated with linoleic acid (160 μM) and were analyzed in a similar fashion. OS15LOX(–) cells did not generate lipoxygenase products when incubated with either arachidonic acid (C) or linoleic acid (not shown). Prostaglandin B₂ was used as an internal standard (IS) for computing extraction efficiency.
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Clones containing active 15-lipoxygenase grew at less than 50% the rate of cells not expressing 15-lipoxygenase. As 15-lipoxygenase activity decreased in these cells over time, their growth rates approached that of the nonexpressing clones. Passage of cells one to two times/week over a 5-month period gradually resulted in substantial loss of activity. Cultures were used at low passage number to minimize variability. Storage of low-passage-number cells permitted ongoing analysis of 15-lipoxygenase in this expression system. The most highly expressing clone, OS15LOX(+), was selected for further characterization, and clone OS15LOX(-), which contained the vector but no insert, was used to determine basal levels of lipoxygenase expression.

Characterization of Expressed 15-Lipoxygenase Activity—

Purified mammalian 15-lipoxygenases have been shown to form a variety of hydroperoxides from arachidonic acid and linoleic acid (26, 27) and cellular peroxidases presumably convert these to their hydroxyl derivatives (6, 7, 28). To identify the enzyme activity expressed by clone OS15LOX(+), cells were incubated with exogenous arachidonic acid or linoleic acid, and the resulting oxygenation products were extracted and analyzed by HPLC. Representative chromatograms are shown in Fig. 2. Incubation of OS15LOX(+)-cells with arachidonic acid resulted in the predominant generation of 15-HETE and lesser amounts of 12-HETE and 15-keto-ETE (Fig. 2, panel A and Table I). Incubation of cells with linoleic acid resulted in predominant generation of 15-HODE and lesser amounts of 13-keto-ODE (Fig. 2, panel B). Cells transformed with the vector alone did not metabolize either linoleic or arachidonic acid (Fig. 2, panel C). All compounds were identified by elution times on RP-HPLC. The amounts of product generated in eight experiments from maximal concentrations of arachidonic acid (160 μM) are shown in Table I. The average ratio of 15-HETE to 12-HETE formed was 8.6 to 1.

In addition, the major peaks coeluting with 15-HETE, 12-HETE, 8S,15S-diHETE, and 13-HODE were subjected to further analysis. Their ultraviolet spectra and elution on SP-HPLC revealed the presence of the characteristic ions at m/z values of 295 (M-111, loss of CH3OH), 311 (M-90, loss of OCH3), 376 (M-15, loss of OCH3), and 389 (M) (Table I). The mass spectrum of the trimethylsilyl ether derivative of 15-HETE methyl ester revealed the presence of the characteristic ions at m/z values of 299 (M-90, loss of Me3SiOH), 311 (M-71, loss of CH3OH), 351 (M-31, loss of OCH3), 376 (M-15, loss of OCH3), and 389 (M) (Table I). The mass spectrum of the trimethylsilyl ether derivative of 13-ΟΗ linoleic acid methyl ester revealed the presence of the characteristic ions at m/z values of 355 (M-71, loss of (CH2)2CH3), 361 (M-90, loss of Me3SiOH), 391 (M-15, loss of CH3OH), and 406 (M). Analysis of the trimethylsilyl ether derivative of 12-HETE identified the characteristic base ion at m/z value of 295 (M-111, loss of CH3OH), 311 (M-90, loss of CH3OH), and 382 (M). The mass spectrum of the trimethylsilyl ether derivative of 13-ΟΗ linoleic acid methyl ester revealed the presence of the characteristic ions at m/z values of 295 (M-111, loss of CH3OH), 311 (M-90, loss of Me3SiOH), 351 (M-31, loss of OCH3), 376 (M-15, loss of OCH3), and 389 (M) (Table I). The mass spectrum of the trimethylsilyl ether derivative of 13-ΟΗ linoleic acid methyl ester revealed the presence of the characteristic ions at m/z values of 355 (M-71, loss of (CH2)2CH3), 361 (M-90, loss of Me3SiOH), 391 (M-15, loss of CH3OH), and 406 (M). The mass spectrum of the trimethylsilyl ether derivative of 13-ΟΗ linoleic acid methyl ester revealed the presence of the characteristic ions at m/z values of 295 (M-111, loss of CH3OH), 311 (M-90, loss of CH3OH), and 382 (M). The mass spectrum of the trimethylsilyl ether derivative of 13-ΟΗ linoleic acid methyl ester revealed the presence of the characteristic ions at m/z values of 295 (M-111, loss of CH3OH), 311 (M-90, loss of Me3SiOH), 351 (M-31, loss of OCH3), 376 (M-15, loss of OCH3), and 389 (M) (Table I).

Several characteristics of product formation by 15-lipoxygenase in OS15LOX(+) cells were determined. Maximal and half-maximal generation of 15-HETE occurred at 160 and 40 μM arachidonic acid, respectively. Product generation was complete within 15 min at 37 °C. Low levels of product were released without loss of vital dye exclusion, although maximal release occurred only after cell viability was significantly decreased. In addition, the same chromatographic profile of products was observed if cells were incubated with substrate in the culture flasks, indicating that the trypsinization before assay did not alter product formation. For example, incubation of cells in monolayer with 20 μM arachidonic acid generated 15% of maximal 15-HETE formation without loss of cell viability. These characteristics are similar to those described for the 15-lipoxygenase of freshly isolated epithelial cells (6).

The formation of 15-HETE was inhibited by preincubation with the inhibitor ETYA in a dose-dependent manner. Approximately 50% of maximal 15-HETE formation occurred at 200 nM ETYA, and complete inhibition occurred at 10 μM. There was no inhibition of enzyme activity seen with indomethacin (10-4 M) which rules out the possibility of HETE formation via a cyclooxygenase mechanism. The effects of both inhibitors are consistent with the described characteristics of native rabbit leukocyte (29) and rabbit reticulocyte 15-lipoxygenase (27).

The transformed cells did not generate 15-lipoxygenase products when challenged with calcium ionophore A23187 (10 μM). Because this ionophore is believed to increase endogenous levels of arachidonic acid, the absence of 15-lipoxygenase activity may be due to the human osteosarcoma cell line not containing enzymes required for endogenous release of substrate (17) or, less likely, to the inability of the methods used in this study to detect minute amounts of product.

Table I

Arachidonic acid lipoxygenase metabolites from clone OS15LOX(+)  

| Peak No. | Retention time (min) | UV absorption (nm) | Compound | Quantity (pmol/10^6 cells) |
|---------|---------------------|-------------------|----------|--------------------------|
|         | RP-HPLC/sp-SP-HPLC  |                   |          |                          |
| 1       | 15.2                | 5.99              | (8S,15S)-Leukotriene | 71 ± 10.7       |
| 2       | 17.2                |                   | (8S,15S)-diHETE | 141 ± 19.9     |
| 3       | 18.9                |                   | (8S,15S)-Leukotriene | 65 ± 12.5      |
| 4       | 20.2                |                   | (8S,15S)-diHETE | 16 ± 3.9       |
| 5       | 24.5                |                   | (14R,15S)-diHETE | 59 ± 8.3       |
| 6       | 35.9                |                   | 15-keto-ETE | 71 ± 11       |
| 7       | 37.7                | 6.59              | 15-HETE | 2817 ± 200     |
| 8       | 40.5                | 6.49              | 12 HETE | 328 ± 20       |

* Average ± S.E., n = 8.
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FIG. 3. Mass spectra of the methyl ester trimethylsilyl ether derivatives of the predominant lipoxygenase metabolites from OS15LOX(+) cells. The diagrams depict major cleavage sites. The fragmentation patterns to the 15-HETE (A), 12-HETE (B), and 13-HODE (C) isolated as in Fig. 2 correspond to those obtained from authentic standards. A phthalate (m/z 149, 167, 279) is present in A and C. In B, the scale between m/z 370 and 395 is magnified x 10 and above 396 it is magnified x 50.

Immunoreactivity of Recombinant 15-Lipoxygenase—Human 5- and 15-lipoxygenase share 40% identity and 60% similarity at the amino acid level (12). To develop a specific antibody to 15-lipoxygenase for expression studies, we synthesized a peptide of 21 amino acids (37-57) according to the predicted primary structure of 15-lipoxygenase. The peptide is from a unique region, relative to 5-lipoxygenase, near the N-terminus of 15-lipoxygenase. Antisera to the peptide (1:50 dilution) hybridized to one major band (70 kDa) in an immunoblot analysis of enriched human leukocyte 15-lipoxygenase (Fig. 4), indicating that the gene products of the 15LOX cDNA and leukocyte 15-lipoxygenase were antigenically related. The basis for the occasional occurrence of a higher molecular weight band is unknown (Fig. 4). The specificity of the immunoreactivity to both bands was evident by the fact that preincubation with synthetic peptide (0.5 mg) but not with an equimolar concentration of bovine serum albumin inhibited the binding. Furthermore, normal rabbit serum in the same dilution did not react with human leukocyte 15-lipoxygenase. The peptide-derived antibody did not hybridize with human 5-lipoxygenase (Fig. 4) or with rabbit reticulocyte lipoxygenase (Fig. 6), reflecting the differences of amino acid sequence in this region of these proteins and confirming the specificity of the resulting antibody.

To obtain antibodies to multiple epitopes of the enzyme, the cDNA was subcloned into the bacterial expression vector pJC264 (Fig. 1B). The bacterial proteins from multiple transformants were analyzed by SDS-PAGE. A band with the expected molecular mass for the CheY-15-lipoxygenase fusion protein (82 kDa) was present in bacteria containing the 15LOX cDNA but not in bacteria transformed with the vector alone or with the vector containing the cDNA in the incorrect orientation (Fig. 5A). This heterologously expressed protein was immunoreactive to the peptide-derived antibody on immunoblots (Fig. 5B). The multiple lower molecular weight bands may be degradation products or partial translation products. The immunoreaction could be inhibited by prein-
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FIG. 5. Expression of CheY-15-lipoxygenase fusion protein in E. coli and the generation of polyclonal antibodies. E. coli were transformed with the expression plasmids pJC264 and pCheY15LOX as described under “Materials and Methods.” Bacterial proteins from colonies with and without 15LOX were separated by SDS-PAGE and were either stained with Coomassie Blue or transferred to nitrocellulose. A, the fusion protein (arrow) was detected on Coomassie Blue-stained gels of colonies containing 15LOX in the correct orientation, CheY15LOX(+), but not of colonies without 15LOX, CheY15LOX(−), or with 15LOX in the incorrect orientation (not shown). B, an immunoblot using the peptide-derived antisera (1:100) demonstrated immunoreactivity to the fusion-protein and to multiple degradation products. No immunoreactivity was observed in CheY15LOX(−) or in CheY15LOX(+/−) if nonimmune sera were used or if immune sera were preincubated with the peptide that was used to generate the antibodies. The recombinant 15-lipoxygenase was purified easily by differential solubility and was used to generate polyclonal antibodies. C, immunoblots were performed with these antibodies on the proteins shown in Fig. 4. The antibodies (1:2000 dilution) recognized one major band in human leukocyte 15-lipoxygenase and did not cross-react to human 5-lipoxygenase. D, the peptide-derived antisera recognized the same band in leukocyte 15-lipoxygenase as the peptide-derived antibody. The antibodies were used to compare the immunoreactivity of recombinant 15-lipoxygenase with that of several cell types. Because reticulocyte lipoxygenase is expressed at significant levels in acute, severe anemia (30) and because patients with this disorder are rare, we used a model of experimental anemia to test of several hypotheses that may relate the enzyme’s molecular size, its antigenicity, its oxygenation of both arachidonic acid and linoleic acid, and its sensitivity to a lipoxygenase inhibitor. Furthermore, the major metabolites produced by the recombinant enzyme have been confirmed by RP-HPLC, SP-HPLC, ultraviolet spectroscopy, and mass spectrometry. These data confirm that the 15LOX cDNA obtained from the human reticulocyte cDNA library does indeed encode human 15-lipoxygenase. The demonstration of active catalytic function of cloned 15-lipoxygenase in an expression system will now permit the testing of several hypotheses that may relate the enzyme’s activity to its structure.

FIG. 6. Immunoreactivity of recombinant 15-lipoxygenase in leukocytes, tracheal epithelial cells, and reticulocytes. A, samples of human leukocyte 15-lipoxygenase (15-LO, 45 μg), rabbit reticulocyte 15-lipoxygenase (10 μg), and the 10,000 × g supernatants (45 μg) of sonicated human tracheal epithelial cells, and osteosarcoma cells containing and not containing 15LOX, OS15LOX(+), and OS15LOX(−), respectively, were separated by SDS-PAGE and stained with Coomassie Blue. B, immunoblots using the CheY-15-LO antibodies (1:3000) showed no cross-reactivity with OS15LOX(−) cells, whereas cross-reactivity to the 15-LO band was detected in OS15LOX(+) cells as well as in leukocytes, tracheal epithelial cells, and reticulocytes. C, immunoblots using the peptide-derived antisera (1:50) identified identical major bands except in rabbit reticulocyte 15-LO where known species-dependent sequence differences account for the nonreactivity.
form 12-lipoxygenation and generates 12-HETE from arachidonic acid. This suggests that the enzyme is multifunctional and is independent confirmation of an earlier observation with purified rabbit reticulocyte lipoxygenase (28); however, these prior experiments could not rule out the possibility of contaminating 12-lipoxygenase. Furthermore, the finding that recombinant 15-lipoxygenase generates 15-HETE and 12-HETE in a ratio of approximately 9:1 suggests that this single enzyme may be responsible for the 12-HETE generated from human airway cells (6) and eosinophils (38). In both cell types and in the OS15LOX(+) cells, arachidonic acid is converted into 15- and 12-HETE in the same ratio.

We observed slower growth of the cell lines actively expressing 15-lipoxygenase. This effect on cell growth may be due to the degradation of mitochondria, as described in reticulocytes (2), or to another effect of lipid peroxidation on other cellular organelles, or to an undescribed action of 15-lipoxygenase. Further study of the OS15LOX(+) cell line may give insight into the intracellular role of 15-lipoxygenase. Furthermore, investigation of how the enzyme is processed in the OS15LOX(+) cells and how it is activated to release 15-lipoxygenase metabolites will yield important information that has been lacking due to the paucity of cell culture systems expressing 15-lipoxygenase. Airway epithelial cells lose enzyme activity rapidly during the first passage of cells. Eosinophils have only recently been maintained in culture for 1–2 weeks, but their 15-lipoxygenase activity has not been described (31). Keratinocytes express 15-lipoxygenase activity only when cocultured with fibroblasts and only in sonicated form (32). Umbilical vein endothelial cells can be cultured for a low passage number and contain small amounts of 15-lipoxygenase activity (33). In contrast, the activity of 15-lipoxygenase in the OS15LOX(+) cells is approximately equal to that of freshly isolated eosinophils, which is 90% that of airway cells and 10–100 times that of other cell types. The OS15LOX(+) cell line, therefore, represents a potential cell system for studying the cellular processing and biological action of human 15-lipoxygenase.

The relationship among 15-lipoxygenases from different tissues is unknown. Isozymes of the soybean 15-lipoxygenase are well described. In addition, the 12-lipoxygenase of bovine platelets has been shown to be immunologically and biochemically distinct from that of leukocytes (34). In contrast, 5-lipoxygenase has been cloned from human placenta (35), HL-60 cells (36), and rat basophilic leukemia cells (37). The primary structure is the same in both human tissues, and there is 90% identity between the rat and the human enzymes.

The most extensively studied mammalian 15-lipoxygenase is the rabbit reticulocyte enzyme which is expressed during the reticulocytosis of anemia (2). While investigators of the rabbit reticulocyte enzyme have presented immunologic and catalytic data that suggest that 15-lipoxygenase is unique to rabbit reticulocytes, it remains possible that in other species the enzyme is less tissue-specific. Alternatively, the tissue catalytic data that suggest that 15-lipoxygenase is unique to tissue along the airway and its epithelium.

Clearly, 15-lipoxygenase activity exists in leukocytes (28), reticulocytes (2, 30), and airway cells (6, 7). The newly developed antibodies described here suggest that the 15-lipoxygenases of these different cell types are antigenerically related. These results extend our prior observation, based on N-terminal sequence data, that the leukocyte and reticulocyte 15-lipoxygenases are structurally related (11). Determination of the full extent of structural similarity among the 15-lipoxygenases of different tissues must await cloning of the cDNAs from tissue-specific libraries but should be facilitated by the human nucleic acid probes and antibodies characterized here. The exact cell types that contain the enzyme within the airway epithelium and inflammatory cells need to be determined by immunocytochemical methods, and these studies can now proceed using the developed antibodies. The antibodies described here are capable of distinguishing cell types with different levels of 15-lipoxygenase expression using immunofluorescent staining.

Finally, the extensive information collected on the biology of the reticulocyte 15 lipoxygenase needs to be examined in terms of its relevance to other cell types. It is likely that the reticulocyte enzyme's action on membrane lipids during cellular degradation and differentiation relates to the biological role of the 15-lipoxygenase in epithelial cells and leukocytes.

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