Presence in human eosinophils of a lysophospholipase similar to that found in the pancreas

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The supernatant fraction from lysed human eosinophils, when separated by gel-filtration chromatography, contains a protein with lysophospholipase activity of approximate molecular mass 74 kDa. This mass differs substantially from the 17 kDa of a previously cloned eosinophil lysophospholipase (Charcot-Leyden crystal protein), but is similar to that reported for a pancreatic enzyme. We have therefore further characterized this pancreatic-like lysophospholipase in human eosinophils. A rabbit polyclonal antibody was produced against a synthetic peptide consisting of amino acids 325–349 from the 74 kDa rat pancreatic lysophospholipase. Western-blot analysis of eosinophil extracts indicate that this antibody recognizes a single 74 kDa band in these preparations. Incubation of the supernatant fraction from

sonified eosinophils with this antibody, followed by precipitation of antibody–antigen complexes with Protein A, removes the majority of the lysophospholipase activity. Indirect immunofluorescence examination with this antibody indicates this protein to be localized to granules of eosinophils and not in other leucocytes. Moreover, reverse transcriptase PCR of polyadenylated RNA from eosinophils and from rat pancreatic tissue with primers to rat pancreatic lysophospholipase resulted in readily detectable 1 kb DNA products in both samples. Sequencing revealed this DNA fragment to be identical with the human pancreatic lysophospholipase cDNA sequence. Taken together, these data indicate that eosinophils contain a lysophospholipase that is similar to the human pancreatic enzyme.

INTRODUCTION

Lysophospholipases are found in most, if not all, cells and some cells have as many as three different forms [1,2]. At least eight different lysophospholipases ranging in mass from 15 to 80 kDa have been isolated and characterized (for a review see ref. [3]). The subcellular distribution of these enzymes is equally diverse: membrane-bound, cytosolic and secreted forms have been reported. Some, such as the membrane-bound 80 kDa lysophospholipase from rat brain, appear to hydrolyse specific lysophospholipids [4] and may attenuate lysophosphatidic acid-mediated signal transduction. In contrast, the 74 kDa pancreatic lysophospholipase is secreted into the intestine, has a broad substrate specificity and is speculated to be involved in digestion [5,6]. Both human and rat pancreatic lysophospholipases have been cloned and have 72 % sequence identity [7–8]. In addition to the pancreas, several other tissues including liver and lactating mammary glands also synthesize this enzyme [10,11]. Thus many different lysophospholipase isoenzymes exist, but our understanding of their tissue distribution is still incomplete.

Eosinophils are rich in lysophospholipase activity [12], which has been attributed to the recently cloned 17 kDa Charcot-Leyden crystal protein [13]. When we detected an eosinophil lysophospholipase that was significantly larger than this protein and similar in mass to the pancreatic lysophospholipase, we characterized it to determine whether eosinophils possess a lysophospholipase that is similar to the pancreatic enzyme.

MATERIALS AND METHODS

Materials

1-[¹⁴C]Palmitoyl-2-hydroxyl-*sn*-glycero-3-phosphocholine (52 mCi/mmol) was from Dupont–New England Nuclear (Boston, MA, U.S.A.). All reagents for peptide and DNA synthesis were obtained from Millipore (Bedford, MA, U.S.A.).

Eosinophil preparation and tissue fractionation

Human eosinophils were obtained from normal donors and were purified from leucocyte fractions (buffy coats) as previously

described [14]. Eosinophils to be lysed were diluted with 5 vol. of Tris (50 mM)-buffered saline containing 1 µg/ml leupeptin (Boehringer-Mannheim, Indianapolis, IN, U.S.A.). Rat pancreatic tissue was excised from male Sprague–Dawley rats (150–200 g). The eosinophils and pancreatic tissue were lysed by sonication (Branson Sonifier; microtip, 4 °C, setting 3). Cell debris was precipitated by centrifugation at 1000 *g* for 10 min and the supernatant was centrifuged at 100 000 *g* to remove all particulate material. The 100 000 *g* supernatant at about 5 mg of protein/ml was used for the experiments described below.

Immunological procedures

A hydrophilic domain with a high degree of sequence similarity in rat and human pancreatic lysophospholipases (ATVDVP-AIDKAKQDVTEEDFYRLVS, amino acids 325–349) was synthesized using F-moc chemistry and a Millipore model 9050 peptide synthesizer. This peptide was purified by reversed-phase HPLC, then cross-linked with glutaraldehyde (3 %, v/v) for 30 min at room temperature. Rabbit polyclonal antibodies were produced and affinity-purified as previously described [15]. The IgG fraction, depleted of antibodies that bound to the synthetic peptide, is referred to as absorbed antibody.

Indirect immunofluorescent examination of eosinophils was performed as described previously [15]. Briefly, human eosinophils were spread on a microscope slide using a cytospin [7 *g* (250 rev./min) for 10 min] then fixed in 2 % paraformaldehyde in PBS. The preparations were incubated with the primary antibody for 1 h at room temperature, washed three times with PBS, then treated with chromotrope 2R (1 %, w/v, in PBS) (Fluka Chemie AG, Bachs, Switzerland) to block autofluorescence. The preparations were then washed three times before incubation with fluorescein-conjugated goat anti-rabbit IgG (Cappel; Chester, PA, U.S.A.) second antibody for 30 min. The cells were washed and mounted in 90 % (v/v) glycerol in 0.1 M Tris, pH 9.0.

Immunoprecipitation experiments were performed on supernatant preparations using affinity-purified antibodies at the

indicated concentrations. Eosinophil and pancreatic tissue supernatant preparations were mixed with the antibodies and incubated at 4 °C for 4 h. Formaldehyde-cross-linked *Staphylococcus aureus* (Immunoprecipitin; Gibco, Grand Island, NY, U.S.A.) was used to precipitate the bound material, as indicated by the manufacturer.

Lysophospholipase assays

The radiometric lysophospholipase assay used is as previously described [1,2]. Briefly, supernatant preparations were incubated with 1-[¹⁴C]palmitoyl-2-hydroxyl-*sn*-glycero-3-phosphocholine, and the 1-[¹⁴C]palmitic acid reaction product was isolated by organic extraction.

Western blotting

SDS/PAGE (10–20 % polyacrylamide gels) was carried out by the method of Laemmli [16]. The proteins were transferred to nitrocellulose using a semidry blotting apparatus at 150 mA for 1 h. Membranes were blocked with 1 % non-fat dry milk in 50 mM Tris-buffered saline. The affinity-purified antibodies were diluted 1:100 in 50 mM Tris/0.05 % Tween 20 in saline (TNT) and incubated with the membranes for 1 h. The membranes were washed and then incubated with alkaline phosphatase-conjugated goat anti-rabbit (1:10000 dilution in TNT) serum for 30 min. 4-Nitro Blue Tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Boehringer-Mannheim) was used as substrate.

Reverse transcriptase PCR

Reverse transcriptase PCR was performed using polyadenylated selected mRNA isolated from human eosinophils and from rat pancreas using a kit and procedures obtained from Biotecx (Houston, TX, U.S.A.). PCR was performed using primers corresponding to nucleotides 494–515 and 1475–1496 of the cDNA of the human pancreatic lysophospholipase [7]. These sequences were conserved in human and rat lysophospholipase cDNAs.

Protein quantification

Proteins were quantified by the method of Bradford [17] using a dye reagent obtained from Bio-Rad (Richmond, CA, U.S.A.) as suggested by the manufacturer. BSA was used as standard.

RESULTS

The possibility that a significant eosinophil lysophospholipase activity was related to pancreatic lysophospholipase was examined by Western blotting (Figure 1). Supernatant preparations from rat pancreas and human eosinophils were separated by SDS/PAGE and transferred to nitrocellulose. The antibody generated to a peptide sequence in rat pancreatic lysophospholipase was used to probe the resulting blots to determine whether eosinophils contain a cross-reactive protein. Results from these experiments indicated that this antibody recognized a single protein in both samples (Figure 1). The mass of this protein was approximately 74 kDa, a value that had previously been reported for the pancreatic lysophospholipase [18].

Indirect immunofluorescence was used to determine the subcellular localization of this immunoreactive protein in human eosinophils. When eosinophils were stained with the antibody to the rat pancreatic lysophospholipase, numerous brightly staining granules were observed in the cytoplasm (Figure 2a). No staining

Figure 1 Western-blot analysis of eosinophil homogenates

Eosinophil (lane A) and rat pancreatic tissue (lane B) supernatant preparations were separated by SDS/PAGE (12 % gel), transferred to nitrocellulose and probed using affinity-purified anti-(pancreatic lysophospholipase) antibody. The molecular-mass standards used were: phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soya-bean trypsin inhibitor (20 kDa) and lysozyme (14 kDa).

Figure 2 Indirect immunofluorescence staining of human leucocytes

(a) Eosinophils were purified from normal human volunteers, fixed in formaldehyde, then stained using affinity-purified anti-(pancreatic lysophospholipase peptide) antibody and visualized using a fluorescein-conjugated goat anti-rabbit IgG antibody. (b) Identical staining of eosinophils with absorbed antisera. (c) Leucocytes viewed by phase-contrast microscopy. (d) The same field of cells as in (c) stained with anti-(pancreatic lysophospholipase) antibody as in (a). Note that only the eosinophils (E) contained immunoreactive material. M, Monocytes; N, neutrophils; L, lymphocytes.

was observed with absorbed antibody (Figure 2b). Leucocytes from buffy coats of human blood were examined by bright field microscopy (Figure 2c). Only the eosinophils in this preparation, identified by their bilobate nuclei visible by bright field microscopy, had brightly staining granules, whereas other cells in the preparation including neutrophils, lymphocytes and macrophages did not appear to contain any immunoreactive material (Figure 2d).

To determine whether the 74 kDa immunoreactive protein in the eosinophils was a lysophospholipase, immunoprecipitation experiments were performed using the anti-(pancreatic lysophospholipase) antibody and *S. aureus*. Treating pancreatic supernatant preparations with the affinity-purified antibody to the pancreatic lysophospholipase completely immuno-

Figure 3 Immunoprecipitation of eosinophil and pancreatic lysophospholipases

Human eosinophils (●) and pancreatic (■) supernatant preparations were incubated with the indicated amounts of affinity-purified anti-(pancreatic lysophospholipase). The antigen-antibody complexes were precipitated using *S. aureus* and the lysophospholipase activity remaining in the supernatant was quantified. In control experiments (○, □), absorbed antisera were used. The data represent the means of four separate experiments with each assay performed in duplicate. Standard errors are less than 15%.

Figure 4 Reverse transcriptase PCR

Primers homologous with the human and rat pancreatic lysophospholipase were used to amplify RNA in both rat pancreas (lane B) and human eosinophils (lane C). Lane A contains λ -HindIII molecular-mass markers.

precipitated this activity in a dose-dependent fashion (Figure 3). Similarly, the antibody precipitated over 80% of the lysophospholipase activity from the eosinophil supernatant. Absorbed antibody did not precipitate activity from either source. These data indicate that eosinophils contain a lysophospholipase that is antigenically related to the enzyme from pancreas.

Nucleic acid probes were also used to establish further at the RNA level that human eosinophils contain a lysophospholipase similar to that found in the pancreas. Using previously published nucleotide sequences for both the rat and human pancreatic lysophospholipases [7–9], we identified two domains (nucleotides 494–515 and 1475–1495) that were completely homologous in the two genes. Polyadenylated RNA was isolated from both rat pancreatic tissue and purified human eosinophils. Reverse transcriptase PCR was performed and a 1 kb PCR product was obtained for both samples, with the human eosinophil fragment being generated on the second round of amplification (Figure 4). The fragment obtained from eosinophils was sequenced and

found to be identical with the previously published sequence from human pancreatic lysophospholipase (results not shown).

DISCUSSION

Eosinophils have long been known to contain large amounts of lysophospholipase activity. It has been proposed that this enzyme performs a protective role for the eosinophil by preventing the cell from being lysed by the large amounts of lysophospholipid present in the plasma membranes of parasites [13,19,20]. Previous studies using histochemical procedures to localize lysophospholipase activity have indicated that most of this activity is in the granules and Golgi apparatus of the human eosinophil [21]. One eosinophil lysophospholipase, the Charcot-Leyden crystal protein with a mass of 17 kDa, has been isolated and cloned [12,13]. This protein is found in the granules, in the cytoplasm and in surprisingly large amounts in the nucleus of the eosinophil [22].

In preliminary studies, we had observed a much larger lysophospholipase (approx. 70 kDa) after gel filtration. Because it had been previously reported that the rat and human pancreas possess a lysophospholipase that was 74 kDa in mass, we investigated whether the large lysophospholipase in the eosinophil was similar to the pancreatic enzyme. In preliminary experiments, we found that the eosinophil lysophospholipase was Ca^{2+} -independent and active in the presence of reducing agents. However, most lysophospholipases have similar enzymic properties. Therefore two independent experimental approaches to this problem were utilized. The first was to determine whether a polyclonal antibody, prepared to the rat pancreatic lysophospholipase, would cross-react with a similar enzyme in eosinophils. The second was to utilize nucleic acid probes and reverse transcriptase PCR.

Western blots of eosinophil supernatant preparations using an antibody to the pancreatic lysophospholipase indicated the presence of a cross-reactive protein that had the same mass as the pancreatic lysophospholipase. Indirect immunofluorescence showed this protein to be localized to the granules of the eosinophil. It was not detected in other leucocytes such as macrophages, neutrophils, lymphocytes or erythrocytes. Furthermore, this antibody immunoprecipitated and neutralized approx. 80% of the lysophospholipase activity in eosinophil supernatant preparations.

In order to explore this phenomenon further, reverse transcriptase PCR was used to detect mRNA. We utilized two primers that amplify a conserved region in both human and rat cDNA sequences, which allowed us to use rat pancreatic mRNA as a positive control. In addition, these primers avoided the highly repetitive 3' end of the message that made amplification of the entire mRNA impossible. A 1 kb DNA fragment was produced from eosinophil RNA by this methodology that, when sequenced, was identical with the previously published human pancreatic lysophospholipase sequence. Taken together, these data indicate that the human eosinophil contains a lysophospholipase that is similar to that secreted by the pancreas.

It has been proposed that the pancreatic lysophospholipase primarily performs a digestive function when released into the intestine. However, the discovery that this protein is also found in milk [11], liver [10] and now the eosinophil suggests that it serves several different functions. Another lipolytic enzyme, phospholipase A_2 , is also released by the pancreas into the digestive system and is thought to have a digestive function. It is now known that the pancreatic phospholipase A_2 is also present in other tissues, including lung and spleen [23–25]. Thus it would

appear that the lipolytic enzymes first identified in the pancreas serve other than digestive functions in other cells and tissues.

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