

Presence of Oxidized Protein Hydrolase in Human Cell Lines, Rat Tissues, and Human/Rat Plasma¹

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Oxidized protein hydrolase (OPH), an 80 kDa serine protease whose activity is inhibited by diisopropyl fluorophosphate (DFP), has been isolated from human erythrocytes [Fujino, T. *et al.* (1998) *J. Biochem.* 124, 1077–1085]. The presence of OPH in various biological samples was examined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting using an anti-OPH antibody raised against OPH purified from human erythrocytes, and by [³H]DFP-labeling and successive SDS-PAGE/fluorography. Solubilized samples of human cell lines including K-562 cells, THP-1 cells and Jurkat cells, and rat tissues including brain, heart, liver, kidney, and testis, inhibited the anti-OPH antibody binding to OPH in ELISA. Immunoblotting of lysates of K-562 cells, THP-1 cells and Jurkat cells showed four immunoreactive protein bands including an 80 kDa protein. Immunoprecipitation of the [³H]DFP-labeled K-562 cell lysate and successive SDS-PAGE/fluorography showed the presence of only the 80 kDa DFP-reactive protein with OPH antigenic activity. The level of the 80 kDa immunoreactive protein in K-562 cells rose as the cells differentiated toward erythrocytes. Immunoblotting of human and rat plasma showed two immunoreactive protein bands, including the 80 kDa protein, and SDS-PAGE/fluorography of [³H]DFP-labeled rat and human plasma showed the presence of only the 80 kDa DFP-reactive protein. The results indicate that OPH is present in a wide variety of biological samples.

Key words: anti-oxidized protein hydrolase antibody, human cell line, oxidized protein hydrolase, rat tissue.

Primary antioxidant defense systems that prevent the generation of free radicals or break radical chain reactions are known to protect living cells from oxidative damage. Secondary antioxidant defense systems including proteases that preferentially degrade oxidatively damaged proteins, have been suggested (1). The preferential degradation of oxidatively damaged proteins is important for the removal of damaged proteins for their renewal. So far, the preferential degradation of oxidatively damaged proteins by several proteases has been demonstrated (2–17). We previously demonstrated the presence of an 80 kDa serine protease, oxidized protein hydrolase (OPH), in human erythrocytes, which preferentially degrades oxidatively damaged membrane proteins (18, 19). This protease is inhibited by diisopropyl fluorophosphate (DFP) (18, 19). It is originally pre-

sent in the cytosol and becomes adherent to cell membranes when the cells are oxidized (19, 20). More recently, OPH has been identified as acylpeptide hydrolase (ACPH) (21). It is not, however, known whether OPH is distributed in biological samples other than human erythrocytes.

In the present study, we examined the presence of OPH in human cell lines, rat tissues and human/rat plasma by enzyme linked immunosorbent assay (ELISA) and immunoblotting using an anti-OPH antibody raised against OPH purified from human erythrocytes, and by [³H]DFP-labeling and successive SDS-PAGE/fluorography. OPH or immunochemically related proteins were present in all the biological samples tested. The OPH present in a wide variety of biological samples may play an important physiological role in the removal of oxidatively damaged proteins.

MATERIALS AND METHODS

Materials—Diisopropyl fluorophosphate (DFP), BSA (globulin free), and protein A Sepharose were purchased from Sigma Chemical (St. Louis, MO). Fetal calf serum (FCS) was from Bio Whittaker (Walkersville, MD); RPMI 1640 medium, penicillin, and streptomycin were from GIBCO Laboratories (Grand Island, NY); o-phenylenediamine was from Tokyo Chemical Industry (Tokyo); Hank's balanced salt solution (HBSS) was from Nissui Seiyaku Corporation (Tokyo); hydrogen peroxide (30%), n-butyric acid, and 2,7-diaminofluorene (DAF) were from Wako Pure Chemical

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Abbreviations: ACPH, acylpeptide hydrolase; DFP, diisopropyl fluorophosphate; DAF, 2,7-diaminofluorene; DPBS (–), Ca²⁺-, Mg²⁺-free Dulbecco's phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; OPH, oxidized protein hydrolase; PVDF, polyvinylidene difluoride.

Industries (Osaka); [1,3-³H]diisopropyl fluorophosphate (³H] DFP) (6.0 Ci/mmol) and EN³HANCE were from NEN Research Products (Boston, MA); ¹²⁵I-goat anti-rabbit IgG was from ICN Biochemicals (Costa Mesa, CA); X-ray film (XAR-5) and hemin were from Eastman Kodak (Rochester, NY); protein A-horseradish peroxidase conjugate was from Bio-Rad Laboratories (Hercules, CA). Polyvinylidene difluoride (PVDF) blotting membranes were obtained from ATTO (Tokyo).

Analysis—Protein levels were determined by the method of Lowry *et al.* (22) using BSA as a reference standard. Spectrophotometric measurement was performed with a Hitachi U-2000 spectrophotometer. Radioactivity was measured on an Aloka LSC 3500 Liquid Scintillation System (Aloka, Tokyo) for β -rays (³H), and an Aloka ARC 2000 Auto Well Gamma System and a Bio-Imaging Analyzer BAS 2000 (Fuji Photo Film, Tokyo) for γ -rays (¹²⁵I). SDS-PAGE was performed according to the method of Laemmli (23) in a discontinuous buffer system with a 7.5 or 10% separating gel and a 4% stacking gel under reducing conditions. Protein samples were solubilized with Laemmli's sample buffer. For fluorographic detection of ³H-radioactivity, the SDS-PAGE gel was treated with EN³HANCE according to the manufacturer's instructions and dried. The radioactivity was visualized on Kodak XAR-5 X-ray film with the aid of an enhancing screen (Lightning Plus) at -80°C.

Purification of Oxidized Protein Hydrolase (OPH) and Preparation of Anti-OPH Antibody—Oxidized protein hydrolase (OPH) was purified from human erythrocytes, according to the method previously described (20). A specific antibody against OPH (anti-OPH antibody) was prepared as described previously (20). Briefly, rabbits [Japanese White, 12 weeks, male from Japan Laboratory Animals Incorporated (Tokyo)] were immunized with purified OPH, and IgG was obtained from the antiserum. The specific antibody was purified from the IgG fraction by affinity chromatography using a sepharose column coupled to thoroughly purified OPH extracted from the 80-kDa protein band of purified OPH in an SDS-PAGE gel.

Cells—K-562 cells (24) and THP-1 cells (25) were obtained from the Japanese Cancer Research Resources Bank of the National Institute of Health Sciences (Tokyo). Jurkat cells (26) was obtained from the Riken Cell Bank of the Institute of Physical and Chemical Research (Saitama). These cell suspensions were cultured in RPMI 1640 medium with 10% heat-inactivated FCS, 50 U/ml penicillin, 50 mg/ml streptomycin, and passaged every 4 or 5 days. After the cells had grown sufficiently, they were washed 3 times with Ca²⁺-, Mg²⁺-Dulbecco's phosphate buffered saline [DPBS (-)] and centrifuged (100 \times g, 4°C, 10 min). Cells were placed on a hemocytometer and counted under a microscope. For the preparation of cell lysates, the cells were shaken in DPBS (-) containing 0.2% Triton X-100 at 4°C for 20 min, and the insoluble materials were removed by centrifugation (27,000 \times g, 4°C, 10 min). The supernatant of the solubilized cells is hereafter called cell lysate.

Rat Tissues—Rats [Wistar, 9 weeks from Japan Laboratory Animals Corporation (Tokyo)] were sacrificed by bleeding. Brain, heart, liver, kidney, and testis were quickly isolated and washed with physiological saline. Each tissue was homogenized in about 10-fold volume of 10 mM potassium phosphate buffer containing 30 mM KCl (pH 7.8) (w/v) with an ice-cooled Potter-type teflon homogenizer. The

homogenate was stored at -80°C under a nitrogen atmosphere before use. For preparation of solubilized homogenates of each tissue, the homogenate was shaken in DPBS (-) containing 0.2% Triton X-100 at 4°C for 20 min, and the insoluble materials were removed by centrifugation (27,000 \times g, 4°C, 10 min). The protein content of the solubilized homogenate was adjusted for use.

Preparation of Human Plasma and Rat Erythrocytes and Plasma, and [³H]DFP-labeling—Fresh human blood was withdrawn intravenously into a syringe with heparin as an anticoagulant; rat blood was withdrawn from the heart into a syringe. Rat erythrocytes were lysed and labeled with [³H]DFP, and the labeled proteins were partially purified according to the method for the preparation of human erythrocyte lysates (20). Blood was centrifuged (650 \times g, 4°C, 10 min), and the supernatant was centrifuged again (1,500 \times g, 4°C, 10 min) to prepare plasma free from erythrocytes, leukocytes and platelets. [³H]DFP-labeling of plasma was performed according to the method previously described (19). Briefly, 20 μ l of a solution of 1 mCi [³H]DFP (0.1 μ l/ml) was added to 10 ml of plasma diluted with DPBS (-) (10 mg protein/ml), and the mixture was incubated at 4°C for 1 h with stirring. To inactivate the remaining serine protease activity, unlabeled DFP was added to the mixture at a final concentration of 2 mM, and the mixture was kept at 4°C for 1 h. The preparation was stored as [³H]DFP-labeled plasma at -80°C.

Differentiation of K-562 Cells Toward Erythrocytes—K-562 cells are caused to differentiate toward erythrocytes by triggering with hemin or *n*-butyric acid (27). A solution of 20 mM hemin in 0.2 M NaOH with 0.6 M Tris-HCl (pH 7.4) or a solution of 1 M *n*-butyric acid sodium salt in HBSS was added to K-562 cell suspensions (1 \times 10⁵ cells to 2 \times 10⁵ cells/ml) to a final concentration of 50 μ M hemin or 1 mM *n*-butyric acid. The cell suspension was cultured for 5 days at 37°C in a 5% CO₂ atmosphere. The differentiated cells were counted after staining the hemoglobin-producing cells using DAF (28). Briefly, the cells were washed 3 times with DPBS (-) and centrifuged (420 \times g, 4°C, 10 min) after each wash. Ten drops of a DAF staining solution prepared by mixing a 1% DAF solution in acetic acid, 0.1 ml of hydrogen peroxide, and 10 ml of 200 mM Tris-HCl (pH 7.0) were added to 2 ml of cell suspension (1 \times 10⁶ cells to 2 \times 10⁶ cells/ml), and the suspension was kept at room temperature for 10 min. Part of the cell suspension was placed on a hemocytometer, and positively colored cells were counted under a microscope to calculate the percentage of differentiated cells.

Detection of OPH Antigens by Enzyme-Linked Immunosorbent Assay (ELISA) and Immunoblotting—ELISA and immunoblotting were performed according to the method previously described (20). Detection of OPH antigens in the sample was performed by competitive inhibition of ELISA in the binding of anti-OPH antibody and OPH. An incubated mixture of anti-OPH antibody and sample was placed in an OPH-coated well, and the plate was incubated. The antibody bound to the plate was measured by incubation with protein A horseradish peroxidase conjugate, followed by the measurement of the bound peroxidase activity using *o*-phenylenediamine and hydrogen peroxide. The brown color was measured by absorbance at 492 nm. The absorbance of a similarly processed control well was subtracted as a blank.

Immunoblotting of the sample was performed as follows. SDS-PAGE of the sample was performed, the protein bands were transferred to a PVDF membrane, and the membrane was incubated first with anti-OPH antibody and then with ^{125}I -labeled goat anti-rabbit IgG as a second antibody. The radioactive bands on the membrane were visualized by radioluminography.

Detection of OPH Antigens in [^3H]DFP-Labeled K-562 Cells—Six microliters of a solution of anti-OPH antibody (30 μg protein) was added to 100 μl of a 50% suspension of protein A Sepharose, and the mixture was incubated at 4°C for 2 h. The protein A Sepharose was recovered and washed with DPBS (–) to prepare the antibody-bound protein A Sepharose. One milliliter of K-562 cell suspension (1×10^6 cells) was sonicated at room temperature for 3 min, and labeled with 10 μl of a solution of 1 mCi [^3H]DFP (0.1 $\mu\text{l}/\text{ml}$) according to the method described for the ^3H -labeling of erythrocytes (19) to prepare about 1 ml of [^3H]DFP-labeled K-562 cell suspension. The whole antibody-bound protein A Sepharose and 0.4 ml of the [^3H]DFP-labeled cell lysate

were mixed, and the mixture was incubated at 4°C for 2 h to immunoprecipitate OPH antigens in the [^3H]DFP-labeled cell lysate. The whole antibody-bound protein A Sepharose to which the OPH antigens adhered was washed with DPBS (–) and subjected to SDS-PAGE. [^3H]DFP-labeled protein bands were detected by fluorography.

RESULTS

OPH was purified from human erythrocytes, and a specific antibody against OPH was prepared by immunizing rabbit with purified OPH. Affinity chromatography of the IgG fraction of the antiserum was then conducted using OPH-coupled Sepharose according to the method previously described (20). The presence of OPH antigens in human myeloleukemia cultured cell lines K-562, THP-1, and Jurkat cells was examined. K-562 cells are a pluripotent stem cell-like cell line that can differentiate into erythrocytes, monocytes, and megakaryocytes (24). THP-1 cells are a monocytic cell line that can differentiate into macrophages

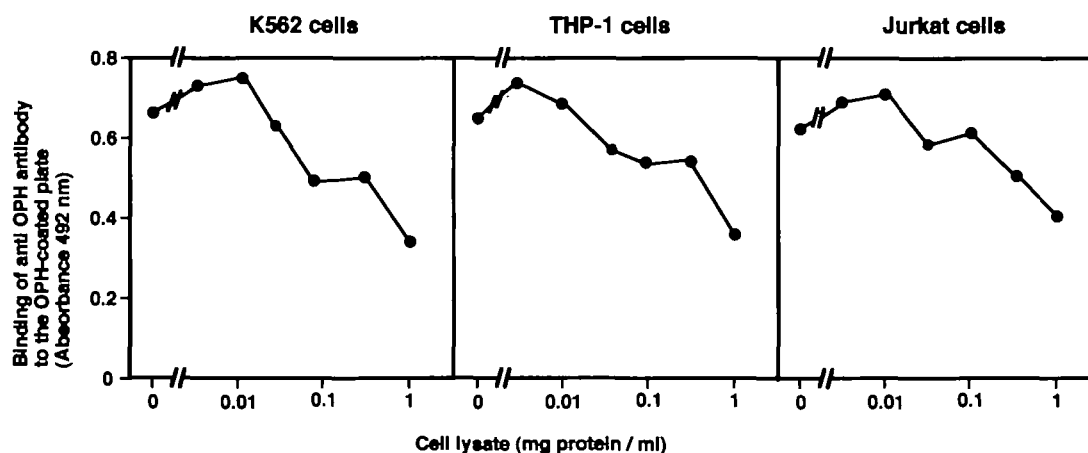


Fig. 1. Immunoreactivity of human cultured cell lines with anti-OPH antibody as detected by competitive inhibition of ELISA. Human cultured cell lines, K-562 cells, THP-1 cells, and Jurkat cells, were lysed with 0.2% Triton X-100/ DPBS (–). The inhibitory

activity of the lysate against the binding of anti-OPH antibody to OPH-coated wells in ELISA was measured at the concentrations indicated.

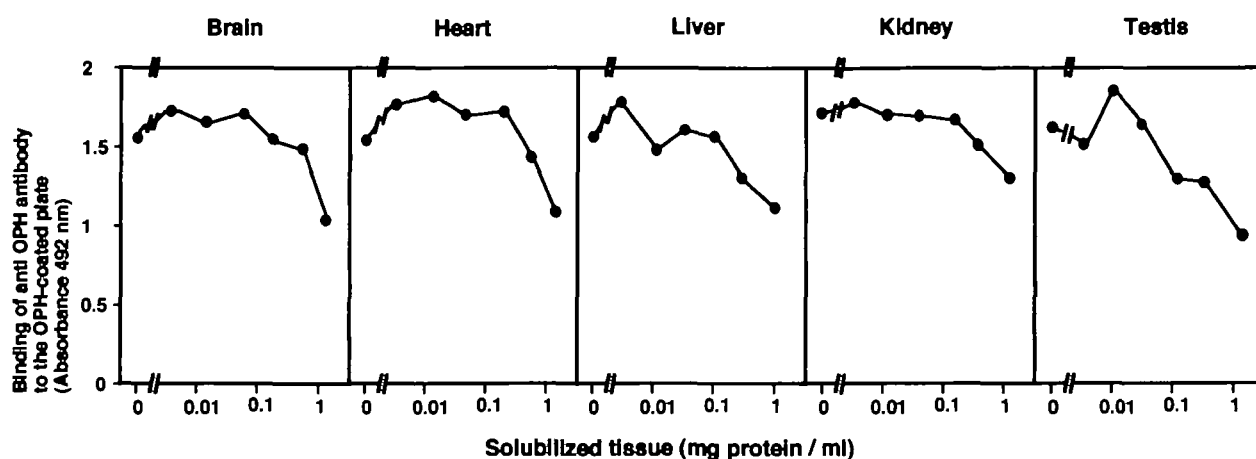


Fig. 2. Immunoreactivity of rat tissues with anti-OPH antibody as detected by competitive inhibition in ELISA. Rat tissues were homogenized and solubilized with 0.2% Triton X-100/

DPBS (–). Their inhibitory activity against the binding of anti-OPH antibody to the OPH-coated wells in ELISA was measured at the concentrations indicated.

(25). Jurkat cells are a T-lymphoid cell line (26). Cells were solubilized in 0.2% Triton X-100. Competitive inhibition of the cell lysate of each cell line against the anti-OPH antibody binding to OPH in ELISA was examined. It was found that each cell line inhibited binding in a dose-dependent manner (Fig. 1). Based on a calibration curve obtained using standard OPH purified from erythrocytes as a competitive inhibitor, the content of OPH or cross-reactive antigens (OPH antigens) in K-562 cells, THP-1 cells and Jurkat cells was estimated to be equivalent to 52, 29, and 19 μg OPH/g cell protein, respectively. The results indicate that OPH antigens are present in these cell lines.

Homogenates of rat tissues including brain, heart, liver, kidney, and testis were solubilized with 0.2% Triton X-100. Competitive inhibition of the solubilized samples of each tissue against anti-OPH antibody binding to OPH in ELISA was examined. It was found that components of each tissue inhibit binding in a dose-dependent manner (Fig. 2). Based on a calibration curve obtained using stan-

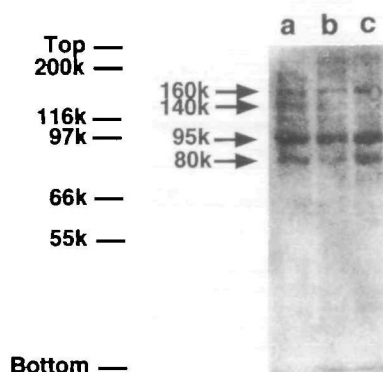


Fig. 3. **Detection of OPH antigens in human cultured cell lines by immunoblotting.** Human cultured cell lines, K-562 cells (a), THP-1 cells (b), and Jurkat cells (c), were lysed with 0.2% Triton X-100/DPBS (-). The lysate of each cell line was subjected to SDS-PAGE under reducing conditions, followed by immunoblotting detection of the cellular proteins that reacted with anti-OPH antibody using ^{125}I -labeled goat anti rabbit IgG as a second antibody. Protein loaded on each lane: 20 μg .

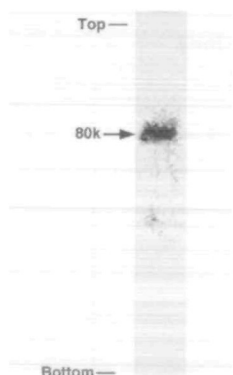


Fig. 4. **Detection of anti-OPH antibody-reactive serine enzyme in ^3H DFP-labeled K-562 cell proteins.** Anti-OPH antibody (30 μg protein) was bound to 50 mg of protein A Sepharose and incubated with ^3H DFP-labeled K-562 cell lysates to immunoprecipitate OPH antigens. The whole protein A Sepharose resin was washed with DPBS (-) and subjected to SDS-PAGE, and the ^3H -DFP-labeled proteins were detected by fluorography.

dard OPH purified from erythrocytes, the content of OPH antigens in rat tissues was estimated to be equivalent to 45.3 (brain), 35.9 (heart), 35.9 (liver), 18.8 (kidney), and 56.3 (testis) μg OPH/g tissue protein. These results indicate that OPH antigens are distributed in various rat tissues.

Immunoblotting of cell lysates of human cultured cell lines was performed using anti-OPH antibody in order to analyze the OPH antigens (Fig. 3). Each cell line showed two major anti-OPH antibody-reactive protein bands at 80 and 95 kDa, and two minor bands at 140 and 160 kDa. Because these bands were not detected using normal rabbit IgG, the bands are specific to the anti-OPH antibody. The OPH antigens in K-562 cells were examined for serine enzyme activity. OPH antigens in ^3H DFP-labeled K-562 cell

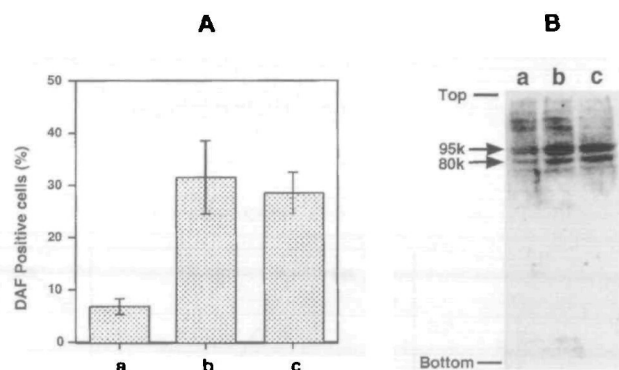


Fig. 5. **Differentiation of K-562 cells and changes in the level of OPH antigens.** Hemin or *n*-butyric acid was added to K-562 cells ($1-2 \times 10^5$ cells/ml) at a final concentration of 50 μM or 1 mM, respectively, and the cells were cultured at 37°C for 5 days under an atmosphere of 5% CO_2 . (A) The percentage of K-562 cells differentiating toward erythrocytes as estimated by staining hemoglobin with DAF. The data are the means of triplicate determinations. a: untreated cells; b: 50 μM hemin-treated cells; c: 1 mM *n*-butyric acid-treated cells. (B) Detection of OPH antigens in K-562 cells differentiated by immunoblotting. K-562 cells were lysed with 0.2% Triton X-100/DPBS (-), and the cell lysates were subjected to SDS-PAGE under reducing conditions, followed by immunoblotting detection using anti-OPH antibody and ^{125}I -labeled goat anti rabbit IgG as a second antibody. Protein loaded in each lane: 30 μg . a: untreated cells; b: 50 μM hemin-treated cells; c: 1 mM *n*-butyric acid-treated cells.

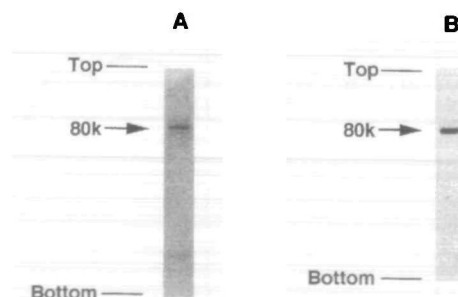


Fig. 6. **Detection of 80 kDa OPH in rat erythrocytes.** ^3H DFP-reactive proteins were partially purified from rat erythrocyte lysate. Partially purified ^3H DFP-labeled proteins were subjected to SDS-PAGE under reducing conditions. (A) Immunoblotting detection using anti-OPH antibody and ^{125}I -labeled goat anti rabbit IgG as a second antibody. Protein loaded on each lane: 2 μg . (B) Detection of ^3H DFP-labeled proteins by fluorography. ^3H -radioactivity loaded in each lane: 3,300 cpm.

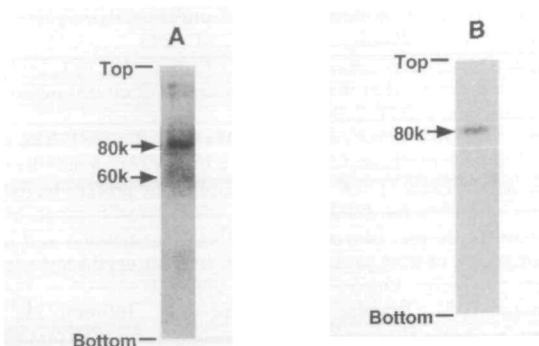


Fig. 7. **Detection of OPH antigenic proteins in human plasma.** (A) Detection by immunoblotting. Human plasma was subjected to SDS-PAGE under reducing conditions, followed by immunoblotting detection of human plasma proteins reactive with anti-OPH antibody using ^{125}I -labeled goat anti rabbit IgG as a second antibody. Protein loaded in each lane: 25 μg . (B) Detection of ^3H DFP-labeled proteins in human plasma. ^3H DFP-labeled human plasma was subjected to SDS-PAGE under reducing conditions, and the ^3H DFP-labeled protein bands were detected by fluorography.

lysates were immunoprecipitated by anti-OPH antibody-bound protein A Sepharose, and the ^3H -labeled OPH antigens were analyzed by SDS-PAGE/fluorography (Fig. 4). Only the 80 kDa DFP-reactive protein was found to be anti-OPH antibody-reactive, indicating that the 80 kDa OPH antigenic protein is an enzyme with active serine residues.

Changes in the level of OPH antigens during the differentiation of K-562 cells to erythrocytes was examined. K-562 cells were cultured with differentiation-inducing agents (27), hemin or *n*-butyric acid, for 5 days. The percentage of hemoglobin-producing cells was calculated by measuring the numbers of cells by DAF staining (28) (Fig. 5A). The percentage of hemoglobin-producing cells, 7% of the untreated cells, increased to 32% in the hemin-treated cells and 28% in the *n*-butyric acid-treated cells. Immunoblotting using anti-OPH antibody (Fig. 5B) showed that the level of the 80 kDa as well as the 95 kDa OPH antigenic protein bands was increased by differentiation with either of the inducing reagents.

Rat erythrocyte lysates were labeled with ^3H DFP, and the DFP-reactive proteins were partially purified. Immunoblotting of the partially purified protein using anti-OPH antibody was performed (Fig. 6A). An immunoreactive 80 kDa protein band was observed. SDS-PAGE/fluorography of the partially purified protein revealed an 80 kDa ^3H -labeled protein band (Fig. 6B). The results indicate that OPH was present in rat erythrocytes.

Immunoblotting of human plasma using anti-OPH antibody (Fig. 7A) showed the presence of 60 and 80 kDa OPH antigens. SDS-PAGE/fluorography of ^3H DFP-labeled human plasma showed the presence of an 80 kDa DFP-reactive protein (Fig. 7B). The 80 kDa protein might be OPH, while the 60 kDa protein had no active serine residues. Immunoblotting of rat plasma using anti-OPH antibody (Fig. 8A) showed the presence of 70 and 80 kDa OPH antigens. SDS-PAGE/fluorography of the ^3H DFP-labeled rat plasma showed the presence of an 80 kDa DFP-reactive protein (Fig. 8B). The 80 kDa protein might be OPH, while the 70 kDa protein had no active serine residues.

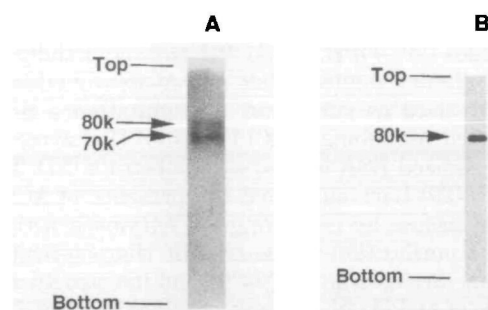


Fig. 8. **Detection of OPH antigenic proteins in rat plasma.** (A) Detection by immunoblotting. Rat plasma was subjected to SDS-PAGE under reducing conditions, followed by immunoblotting detection of rat plasma proteins reactive with anti-OPH antibody using ^{125}I -labeled goat anti rabbit IgG as a second antibody. Protein loaded in each lane: 25 μg . (B) Detection of ^3H DFP-labeled proteins in rat plasma. ^3H DFP-labeled rat plasma was subjected to SDS-PAGE under reducing conditions, and the ^3H DFP-labeled protein bands were detected by fluorography.

DISCUSSION

During the course of the investigation of the enzymatic removal of oxidatively damaged proteins from human erythrocyte membranes, we found an 80 kDa serine protease in the membranes of oxidized erythrocytes and unoxidized erythrocyte cytosol that preferentially degraded oxidatively damaged proteins. This enzyme was termed oxidized protein hydrolase (OPH) (18–21). In the present study, OPH and immunochemically related proteins were detected in human cultured cell lines, rat tissues and plasma, and human plasma by ELISA and immunoblotting using an anti-OPH antibody raised against OPH purified from human erythrocytes, and/or by fluorography of ^3H -DFP-labeled proteins. The 80 kDa protein detected in the present study was both immunoreactive to anti-OPH antibody and labeled by DFP, and therefore was concluded to be OPH.

OPH antigens other than the 80 kDa protein were detected in human cell lines, including K-562 cells, THP-1 cells, and Jurkat cells, and rat and human plasma by immunoblotting. These proteins may be OPH-family proteins without reactive serine residues. Three OPH antigens with larger molecular weights were detected in human cultured cell lines, and single OPH antigens with smaller molecular weights were detected in human and rat plasma. It is not known whether these OPH-related proteins are the precursors or the degradation products of OPH.

Immunoblotting showed that the level of OPH in K-562 cells increases with cell differentiation toward erythrocytes. Unnecessary oxidatively damaged proteins generated in the cells during differentiation may be removed by OPH. OPH is present in mature erythrocytes so as to degrade and remove unnecessary oxidatively damaged proteins from cells in circulation (20). OPH was found to be present in extracellular plasma, but the role of extracellular OPH is not known.

More recently, OPH obtained from human erythrocytes was found to be identical to a known acylpeptide hydrolase (ACPH) by comparison of their amino acid sequences and enzymatic activities (21). ACPH, first isolated from rat liver

by Tsunasawa *et al.* (29) is a serine exopeptidase that releases an acylated N-terminal amino acid from N-acylated peptides (29). OPH has ACPH enzyme activity when N-acetyl-L-alanine *p*-nitroanilide and N-acetylmethionyl L-alanine are used as substrates, and glutathione S-transferase-tagged recombinant ACPH has OPH enzyme activity when oxidized BSA is used as a substrate (21). Tsunasawa *et al.* (29) have suggested the presence of ACPH in various rat tissues by measuring the enzymatic activity of ACPH. The purification and enzymatic characterization of ACPH from various origins (29–35), and the structural considerations of ACPH (36–42) have been accomplished. However, the physiological functions of ACPH has not yet been elucidated, whereas a possible role of ACPH in the co-translational or post-translational modification of the nascent polypeptide chain has been discussed (34), and the possible use of ACPH for structural studies on blocked peptide or protein substrates has been evaluated (29, 34).

Earlier studies that surveyed ACPH activity have shown that ACPH is distributed in a wide variety of tissues (29, 35); the present study using anti-OPH antibody and DFP-labeling shows that OPH and immunochemically related proteins are also widely distributed in a variety of cells and tissues. Because OPH is found to be identical to ACPH, the physiological role of the enzyme is extended, and it may play an important role in the removal of oxidatively damaged proteins from cells and tissues.

REFERENCES

- Davies, K.J.A. (1986) Intracellular proteolytic systems may function as secondary antioxidant defenses: An hypothesis. *Free Rad. Biol. Med.* **2**, 155–173
- Goldberg, A.L. and Boches, F.S. (1982) Oxidized proteins in erythrocytes are rapidly degraded by the adenosine triphosphate-dependent proteolytic system. *Science* **215**, 1107–1109
- Fagan, J.M., Waxman, L., and Goldberg, A.L. (1986) Red blood cells contain a pathway for the degradation of oxidant-damaged hemoglobin that does not require ATP or ubiquitin. *J. Biol. Chem.* **261**, 5705–5713
- Davies, K.J.A. and Goldberg, A.L. (1987) Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. *J. Biol. Chem.* **262**, 8220–8226
- Davies, K.J.A. and Goldberg, A.L. (1987) Proteins damaged by oxygen radicals are rapidly degraded in extracts of red blood cells. *J. Biol. Chem.* **262**, 8227–8234
- Davies, K.J.A. and Goldberg, A.L. (1987) Protein damage and degradation by oxygen radicals. General aspect. *J. Biol. Chem.* **262**, 9895–9901
- Salo, D.C., Lin, S.W., Pacifici, R.E., and Davies, K.J.A. (1988) Superoxide dismutase is preferentially degraded by a proteolytic system from red blood cells following oxidative modification by hydrogen peroxide. *Free Rad. Biol. Med.* **5**, 335–339
- Davies, K.L.A. and Lin, S.W. (1988) Degradation of oxidatively denatured proteins in *Escherichia coli*. *Free Rad. Biol. Med.* **5**, 215–223
- Davies, K.L.A. and Lin, S.W. (1988) Oxidatively denatured proteins are degraded by an ATP-independent proteolytic pathway in *Escherichia coli*. *Free Rad. Biol. Med.* **5**, 225–236
- Pacifici, R.E., Salo, D.C., and Davies, K.J.A. (1989) Macroxypoteinase (M.P.O.): A 670 kDa proteinase complex that degrades oxidatively denatured proteins in red blood cells. *Free Rad. Biol. Med.* **7**, 521–536
- Sacchetta, P., Battista, P., Santarone, S., and Cola, D.D. (1990) Purification of human erythrocyte proteolytic enzyme responsible for degradation of oxidant-damaged hemoglobin. Evidence for identifying as a member of the multi-catalytic proteinase family. *Biochim. Biophys. Acta* **1037**, 337–343
- Fagan, J.M. and Waxman, L. (1991) Purification of a protease in red blood cells that degrades oxidatively damaged haemoglobin. *Biochem. J.* **277**, 779–786
- Murakami, T., Hatanaka, M., and Murachi, T. (1981) The cytosol of human erythrocytes contains a highly Ca²⁺-sensitive thiol protease (calpain I) and its specific inhibitor protein (calpastatin). *J. Biochem.* **90**, 1809–1816
- Yamamoto, K. and Marchesi, V.T. (1984) Purification and characterization of acid proteinase from human erythrocyte membranes. *Biochim. Biophys. Acta* **790**, 208–218
- Yamamoto, K., Takeda, M., Yamamoto, H., Tatsumi, M., and Kato, Y. (1985) Human erythrocyte membrane acid proteinase (EMAP): Sideness and relation to cathepsin D. *J. Biochem.* **97**, 821–830
- Yamamoto, K., Yamada, M., and Kato, Y. (1989) Age-related and phenylhydrazine-induced activation of the membrane-associated cathepsin E in human erythrocytes. *J. Biochem.* **105**, 114–119
- Takeda-Ezaki, M. and Yamamoto, K. (1993) Isolation and biochemical characterization of procatepsin E from human erythrocyte membranes. *Arch. Biochem. Biophys.* **304**, 352–358
- Beppu, M., Inoue, M., Ishikawa, T., and Kikugawa, K. (1994) Presence of membrane-bound proteinases that preferentially degrade oxidatively damaged erythrocyte membrane proteins as secondary antioxidant defense. *Biochim. Biophys. Acta* **1196**, 81–87
- Fujino, T., Ishikawa, T., Inoue, T., Beppu, M., and Kikugawa, K. (1998) Characterization of membrane-bound serine protease related to degradation of oxidatively damaged erythrocyte membrane proteins. *Biochim. Biophys. Acta* **1374**, 47–55
- Fujino, T., Tada, T., Beppu, M., and Kikugawa, K. (1998) Purification and characterization of a serine protease in erythrocyte cytosol that is adherent to oxidized membranes and preferentially degrades proteins modified by oxidation and glycation. *J. Biochem.* **124**, 1077–1085
- Fujino, T., Watanabe, K., Beppu, M., Kikugawa, K., Sasuda, H. (2000) Identification of oxidized protein hydrolase of human erythrocytes as acylpeptide hydrolase. *Biochim. Biophys. Acta*, in press
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
- Koeffler, H.P. and Golde, D.W. (1980) Human myeloid leukemia cell lines: A review. *Blood* **56**, 344–350
- Tsuchiya, S., Kobayashi, Y., Goto, Y., Okumura, M., Nakae, S., Konno, T., and Tada, T. (1982) Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. *Cancer Res.* **42**, 1530–1536
- Schneider, U., Schwenk, H.U., and Bornkamm, G. (1977) Characterization of EBV-genome negative 'null' and 'T' cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. *Int. J. Cancer* **19**, 521–526
- Cioe, L., McNab, A., Hubbel, H.R., Meo, P., Curtis, P., and Rovera, G. (1981) Differential expression of the globine genes in human leukemic K562(S) cells induced to differentiate by hemin or butyric acid. *Cancer Res.* **41**, 237–243
- Worthington, R.E., Bossie-Codreanu, J., and Van-Zant, G. (1987) Quantitation of erythroid differentiation in vitro using a sensitive colorimetric assay for hemoglobin. *Exp. Hematol.* **15**, 85–92
- Tsunasawa, S., Narita, K., and Ogata, K. (1975) Purification and properties of acylamino acid-releasing enzyme from rat liver. *J. Biochem.* **77**, 89–102
- Gade, W. and Brown, L.J. (1978) Purification and partial characterization of α -N-acylpeptide hydrolase from bovine liver. *J. Biol. Chem.* **253**, 5012–5018

31. Jones, W.M. and Manning, J.M. (1985) Acylpeptide hydrolase activity from erythrocytes. *Biochem. Biophys. Res. Commun.* **128**, 933–940
32. Jones, W.M., Manning, L.R., and Manning, J.M. (1986) Enzymatic cleavage of the blocked amino terminal residues of peptides. *Biochem. Biophys. Res. Commun.* **139**, 244–250
33. Kobayashi, K. and Smith, J.A. (1987) Acyl-peptide hydrolase from rat liver, characterization of enzyme reaction. *J. Biol. Chem.* **262**, 11435–11446
34. Jones, W.M. and Manning, J.M. (1988) Substrate specificity of an acylamino peptide that catalyzes the cleavage of the blocked amino termini of peptides. *Biochim. Biophys. Acta* **953**, 357–360
35. Radhakrishna, R.G. and Wold, F. (1989) Purification and characterization of an *N*-acylaminoacyl-peptide hydrolase from rat muscle. *J. Biol. Chem.* **264**, 11076–11081
36. Kobayashi, K., Lin, L.W., Yeadon, J.E., Klickstein, L.B., and Smith, J.A. (1989) Cloning and sequence analysis of a rat liver cDNA encoding acyl-peptide hydrolase. *J. Biol. Chem.* **264**, 8892–8899
37. Mitta, M., Asada, K., Uchiyama, Y., Kunizuka, F., Kato, I., Sakiyama, F., and Tsunasawa, T. (1989) The primary structure of porcine liver acylamino acid-releasing enzyme deduced from cDNA sequences. *J. Biochem.* **106**, 548–551
38. Jones, W.M., Scaloni, A., Bossa, F., Popowicz, A.M., Schneewind, O., and Manning, J.M. (1991) Genetic relationship between acylpeptide hydrolase and acylase, two hydrolytic enzymes with similar binding but different catalytic specificities. *Proc. Natl. Acad. Sci. USA* **88**, 2194–2198
39. Scaloni, A., Jones, W.M., Barra, D., Pospischil, M., Sassa, S., Popowicz, A., Manning, L.R., Schneewind, O., and Manning, J.M. (1992) Acylpeptide hydrolase: Inhibitions and some active site residues of the human enzyme. *J. Biol. Chem.* **267**, 3811–3818
40. Miyagi, M., Sakiyama, F., Kato, I., and Tsunasawa, S. (1995) Complete covalent structure of porcine liver acylamino acid-releasing enzyme and identification of its active site serine residue. *J. Biochem.* **118**, 771–779
41. Mitta, M., Ohnogi, H., Mizutani, S., Kato, I., Sakiyama, F., and Tsunasawa, S. (1996) The nucleotide sequence of human acylamino acid-releasing enzyme. *DNA Res.* **3**, 31–35
42. Mitta, M., Miyagi, M., Kato, I., and Tsunasawa, S. (1998) Identification of the catalytic triad residues of porcine liver acylamino acid-releasing enzyme. *J. Biochem.* **123**, 924–931