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QUANTITATIVE DETERMINATION OF HUMAN ALDOSE REDUCTASE BY ENZYME-LINKED IMMUNOSORBENT ASSAY

IMMUNOASSAY OF HUMAN ALDOSE REDUCTASE

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Abstract—An antibody-sandwich enzyme-linked immunosorbent assay (ELISA) for evaluating tissue levels of aldose reductase was developed using a polyclonal antibody prepared against the recombinant enzyme expressed in a baculovirus system. The specificity of this antibody to aldose reductase was verified by immunoprecipitation, immunoblotting and ELISA. The polyclonal antibody did not cross-react with human aldehyde reductase, an enzyme in the same aldo-keto reductase family structurally and functionally related to aldose reductase. The sensitivity and specificity of this assay method enabled direct determination of aldose reductase level in various human tissues including the erythrocyte. The highest level of aldose reductase was detected in the kidney medulla among tissues investigated. More than a 2-fold variability in the erythrocyte aldose reductase was demonstrated among healthy individuals, indicating the heterogeneity of this enzyme expression in a human population. This assay system may be useful for direct measurement of the level of tissue aldose reductase in conjunction with the evaluation of the efficacy of aldose reductase inhibitors prescribed for the treatment of diabetic complications.

Aldose reductase (EC 1.1.1.21) is a member of the monomeric NADPH-dependent aldo-keto reductase family. This enzyme catalyses the reduction of various aldehydes, including the aldehyde form of glucose, to the corresponding sugar alcohol, sorbitol. Sorbitol is subsequently converted to fructose by sorbitol dehydrogenase which, together with aldose reductase, constitutes the sorbitol (polyol) pathway. Little is known about the physiological role of this pathway. In kidney tubular cells, however, sorbitol may be used to increase cellular tonicity by acting as an osmolyte in the process of urine concentration [1].

A growing body of evidence has implicated aldose reductase in the pathogenesis of various diabetic complications such as neuropathy [2–4], retinopathy [5–7] and cataracts [8]. Although the exact mechanisms by which aldose reductase initiates these complications are not fully understood, in ocular cataracts the osmotic stress caused by the intracellular accumulation of sorbitol is thought to play a primary role in the development of lens opacity. Consequently numerous enzyme inhibitors have been explored as possible therapeutic agents for diabetic patients, some of which are already clinically prescribed in a few countries.

Recent studies on aldose reductase isolated from human tissues suggest significant variation in the endogenous level of this enzyme among individuals. Marked variability in the reductase activity was reported for enzyme preparation extracted from human placentas [9]. Aldose reductase isolated from erythrocytes exhibited a nearly 3-fold variation in activity among diabetic patients [10]. Since the production of sorbitol is believed to be a critical step in the development of diabetic complications, evaluation of the heterogeneity of aldose reductase levels among diabetic patients may be as vital as measurement of plasma glucose levels. In addition, it is possible that the efficacy of aldose reductase inhibitors on diabetic patients may be affected by the variable level of the tissue enzyme.

Currently the tissue aldose reductase level is estimated either by measuring the enzyme activity recovered in the purified protein fraction, or by assessing the amount of sorbitol present in the tissue. Due to the coexistence of aldehyde reductase (EC 1.1.1.2) in most of the human tissues, which shares overlapping substrate specificity with aldose reductase, it is necessary to fractionate these enzymes prior to assay. This procedure is not only laborious but requires a substantial amount of tissue sample for the fractionation [9, 10]. On the other hand determination of sorbitol levels lacks specificity since this sugar alcohol is not generated solely by aldose reductase [11]. To measure the precise level of this enzyme in the small amount of specimen obtained from human subjects, we have developed a sensitive immunoassay method utilizing antibodies raised against recombinant enzyme generated in a baculovirus expression system [12, 13].

MATERIALS AND METHODS

Preparation of antibodies. Polyclonal antibodies

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were raised in adult female rabbits (Japan White, 2.0–2.5 kg body weight). Multiple intradermal injections were given to rabbits anesthetized with Ketalar 50 (Parke-Davis) and Rompun (Bayer). The antigen injected was either 0.5–1.0 mg purified recombinant human aldose reductase or 200 μg synthetic peptide conjugated to keyhole limpet hemocyanin mixed with complete Freund's adjuvant. The purified synthetic peptide named C-P, corresponding to the sequence of the carboxyl terminal end of human aldose reductase CTSHKDYPFHEEF [12], was commercially synthesized (Mitani Sangyo, Tokyo, Japan). Following the NIH guidelines, rabbits were boosted with the same amount of antigen in adjuvant, three times at intervals of 2–3 weeks. For monoclonal antibodies, BALB/c mice were immunized with 50 μg of purified recombinant enzyme mixed with complete Freund's adjuvant, followed by several booster injections. Spleen cells of immunized mice were fused with NS1 myeloma cells as previously described [14] and antibodies produced in hybridoma media were screened by the direct enzyme-linked immunosorbent assay (ELISA*) [15].

Polyclonal antibodies in rabbit antisera and a monoclonal antibody produced in mouse ascites fluid were affinity purified using Ampure PA kit (Amersham, Bucks, U.K.) according to the manufacturer's protocol.

Immunoprecipitation and immunoblot analysis. Purified IgG fractions of preimmune or immunized rabbit serum containing anti-aldose reductase polyclonal antibody were coupled to CNBr-activated Sepharose 4B (Pharmacia LKB, Piscataway, NJ, U.S.A.) according to the manufacturer's instructions. Frozen stored human testis was homogenized in 2–3 vol. of Tris–saline–azide solution (TSA; 10 mM Tris–HCl, 140 mM NaCl, 0.025% NaN_3, pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, and 0.2 U/mL trypsin inhibitor (the Sigma Chemical Co., T-9003). The soluble fraction isolated by centrifugation at 100,000 *g* for 30 min was precleared with quenched control Sepharose, and incubated with antibody-coupled Sepharose at 4° for 60 min. The Sepharose beads were then washed twice with 0.1% Triton X-100 in TSA, and once with TSA alone. After the final wash with 50 mM Tris–HCl (pH 6.8), the bound protein was dissociated from the beads by incubation in sodium dodecyl sulfate (SDS) sample buffer at 100° for 5 min [16]. Following electrophoresis in a 12.5% SDS–polyacrylamide gel [17], the precipitated protein was detected by silver staining [18].

For immunoblot analysis, protein separated in SDS–polyacrylamide gels was transferred electrophoretically to polyvinylidene difluoride membrane (Immunobilon, Millipore Co.) in 25 mM Tris–HCl, 150 mM glycine and 10% methanol buffer, at pH 8.3 [19]. Membranes blocked with 5% instant powdered milk in phosphate-buffered saline (PBS) were incubated with 1 μg/mL of anti-aldose reductase or anti-C-P polyclonal antibody diluted in the blocking solution containing 0.1% Triton X-100. Bound antibody on the membrane was detected using 1/1000 dilution of peroxidase-conjugated swine anti-rabbit immunoglobulins (DAKOPATTS a/s) and ECL western blotting detection system (Amersham).

Preparation of tissue samples. Among human tissues investigated, sciatic nerve, heart muscle, testis, liver, spleen and lung were obtained at autopsy within 6 hr of death and stored frozen at −70° until use. Except for the cornea from the patient of cornical cornea, all other samples were from normal sections of surgically removed specimen for curative purpose. These samples were immediately frozen in liquid nitrogen and stored at −70°. The thawed tissues were homogenized in 3–10 vol. of TSA solution containing 1 mM phenylmethylsulfonyl fluoride. The supernatant fraction isolated by two cycles of centrifugation at 15,000 *g* for 30 min, was used for the subsequent imunoassay. Protein concentration was determined by the method of Bradford [20].

For human erythrocyte, approximately 1 mL of blood was drawn from healthy adults after informed consent was obtained. Biannual checkups of the healthy individuals indicated normal plasma glucose level and no glycosuria for more than 2 years. Heparinized whole blood was transferred to a tube containing 1 mL of acid–citrate–dextrose solution (23 mM citric acid, 45 mM sodium citrate, 82 mM dextrose), and stored at 4° for less than 7 days. In preliminary experiments no significant change in aldose reductase level had been found during 7 days of storage. Plasma and buffy coat were removed from the sample by centrifugation at 1,000 *g* for 10 min. The resultant erythrocytes, washed twice with 10 vol. of ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3), were hemolysed by adding an equal volume of 20 mM phosphate buffer (pH 7.0) and by two cycles of freezing and thawing using a dry ice acetone bath. The supernatant fraction of the hemolysate was obtained by centrifugation at 15,000 *g* for 15 min. and stored at −80° for the following immunoassay. Hemoglobin concentration in the hemolysate was determined by the hemoglobincyanide method [21].

Antibody-sandwich ELISA. In the antibody-sandwich ELISA, antibody is first immobilized on plastic plate. This antibody captures the antigen. A sandwich complex is formed following the addition of a second antibody conjugated to an enzyme for detection. Purified antibodies were conjugated to alkaline phosphatase (ALP) using Immuno-Link AP labelling kit (Cambridge Research Biochemicals, Northwich, U.K.) according to the manufacturer's instructions. Immunoplates (Maxisorp F96, Nunc) were coated with 50 μL of purified antibodies (5 μg/mL) diluted in 50 mM carbonate buffer (pH 9.6) at room temperature for 2 hr or at 4° overnight. Plates were washed three times with washing solution (0.9% NaCl, 0.05% Tween 20) and blocked with 100 μL of blocking buffer containing 170 mM H_3BO_3, 120 mM NaCl, 0.05% Tween 20, 1 mM ethylenediaminetetraacetic acid, 0.25% bovine serum albumin (Sigma A-7030) and 0.05% NaN_3

* Abbreviations: ELISA, enzyme-linked immunosorbent assay; TSA, Tris–saline–azide solution; SDS, sodium dodecyl sulfate; ALP, alkaline phosphatase; PBS, phosphate-buffered saline.

(pH 8.5) for 30 min at room temperature. Fifty microliters of antigen solution diluted with blocking buffer were placed in each well and incubated at 4° for 18 hr with constant agitation. Following three washes of the plates with washing solution, 50 μL of ALP-conjugated antibody (4.5 μg/mL) in blocking buffer was placed and incubated at room temperature for 2 hr with constant agitation. Following three washes with washing solution and two washes with deionized water, antigen–antibody complex formed in each well was detected by adding 75 μL of the substrate solution containing 1 mg/mL *p*-nitrophenyl phosphate and 1 mM levamisole dissolved in diethanolamine–HCl buffer (10 mM diethanolamine–HCl, 0.5 mM $MgCl_2$, pH 9.8). Hydrolysis of the substrate was measured at 405 nm with a plate reader (Labsystems Multiskan Bichromatic) at the time when the highest O.D. reading in the plate reached 1.0–1.5. Known amounts of a standard antigen of purified recombinant aldose reductase diluted with blocking buffer were added to wells of each plate to construct a standard curve fitted by linear regression. Each unknown sample was serially diluted in duplicate, and the concentration of aldose reductase was interpolated by applying the O.D. readings to the respective standard curves.

RESULTS

Preparation of antibodies for sandwich ELISA

Antibodies were raised against purified recombinant human aldose reductase and a synthetic peptide C–P. Although the identity of the recombinant human enzyme was previously verified [13], a recent study using a baculovirus system for recombinant aldose reductase expression reported the co-purification of an aldose reductase-like enzyme originating from the host insect cells,

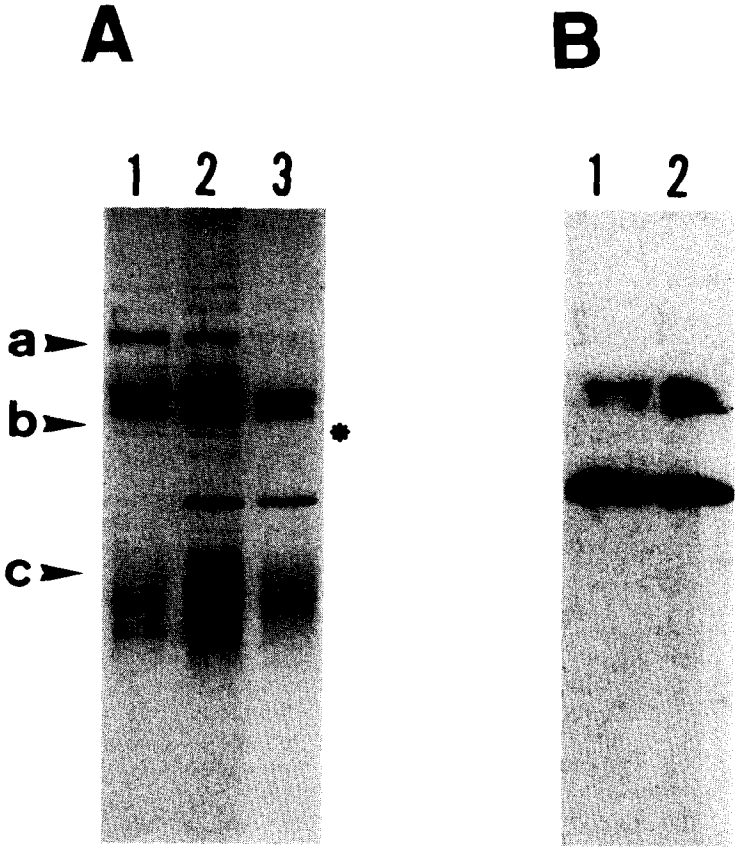

Fig. 1. Specificity of ARAb verified by immunoprecipitation. (A) Silver staining of the precipitates separated on a 12.5% SDS–polyacrylamide gel. The soluble fraction of human testis (lanes 1 and 2) or purified recombinant aldose reductase (lane 3) was immunoprecipitated with either preimmune IgG (lane 1) or ARAb (lanes 2 and 3). (B) Immunoblot of the same gel as in A. The protein precipitated from testis (lane 1) or purified aldose reductase (lane 2) with ARAb was separated on a polyacrylamide gel, transferred to a membrane and detected with ARAb. Similar results were obtained for the immunoblot detected with CPAb. Approximately one third of the total immunoprecipitates originated from 0.7 g wet/wt of testis or 3 μg of purified aldose reductase was loaded on each lane. Arrowheads denote the migration of marker proteins (a, 69K; b, 46K; c, 30K). The doublet bands present in all samples between (a) and (b) presumably derive from rabbit IgG. An asterisk denotes nonspecific protein precipitated with rabbit IgG.

Spodoptera frugiperda (SF9), if the recombinant protein was extracted directly from the cell homogenate [22]. We therefore examined whether insect cell protein contaminated our recombinant enzyme preparation. When culture medium of SF9 cells infected with wild-type baculovirus (*Autographa californica* nuclear polyhedrosis virus) was subjected to purification as described previously [13], the protein fraction eluted from the affinity column corresponded to not more than 6% of the total protein recovered from the culture medium of recombinant virus infected cells under the same experimental conditions. The specific activity of the protein fraction derived from SF9, determined as DL-glyceraldehyde reducing activity per mg protein, was less than 7% of the purified recombinant aldose reductase (unpublished observation).

Initially the applicability to sandwich ELISA was investigated using three different antibodies, anti-aldose reductase polyclonal antibody (ARAb), anti-C-P polyclonal antibody (CPAb) and anti-aldose reductase monoclonal antibody (A6). Plates were coated with either of these antibodies and incubated with 3–50 ng/mL of purified recombinant enzyme. Bound antigen was detected with either ARAb and peroxidase-conjugated anti-rabbit immunoglobulins, or ALP-conjugated ARAb. The highest O.D. reading in each antigen concentration was demonstrated in the wells coated with ARAb (data not shown).

To determine the applicability of the labeled antibodies, ALP-conjugated ARAb and CPAb were examined using wells coated with ARAb. Compared with ALP–CPAb, more than 5-fold sensitivity was demonstrated with ALP–ARAb. The lowest limit of detection with ALP–ARAb was approximately 0.5 ng/mL (data not shown).

Specificity of ARAb to aldose reductase in human tissues

Specificity of ARAb was subsequently defined for the usage in immunoassay. Figure 1 demonstrates immunoprecipitation of aldose reductase in the human testis with ARAb-coupled Sepharose. The immunoprecipitated proteins were separated in a SDS–polyacrylamide gel and either stained with silver (Fig. 1A) or electroblotted (Fig. 1B). Silver staining of the gel clearly exhibited a band identical to the size of recombinant human aldose reductase immunoprecipitated under the same experimental

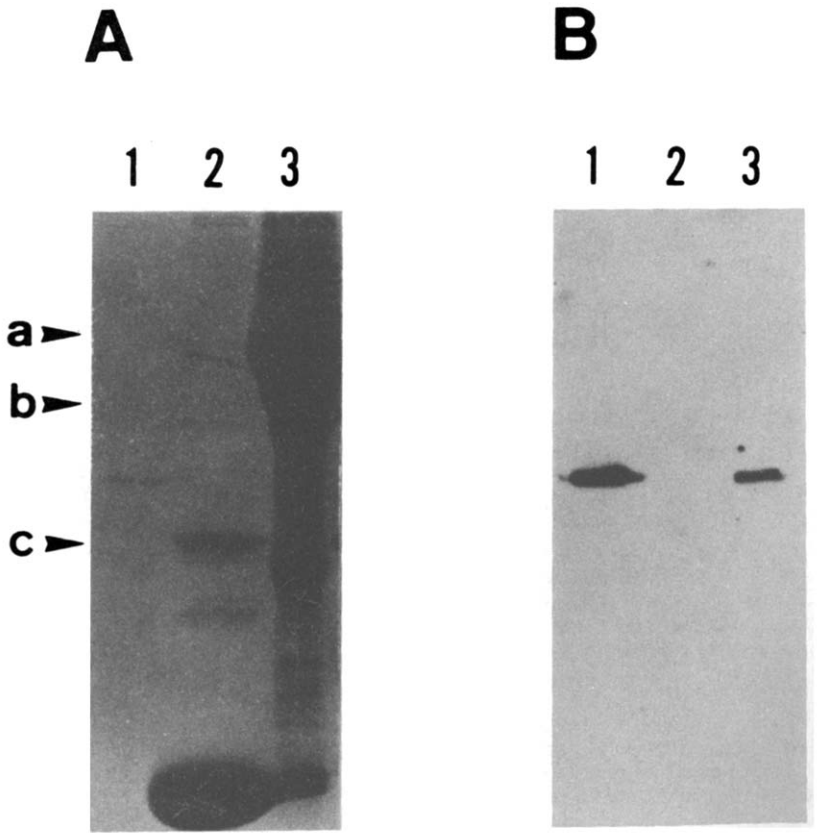

Fig. 2. Specificity of ARAb verified by immunoblot analysis of human tissues. (A) Coomassie Blue staining of purified recombinant aldose reductase (1 μg, lane 1), erythrocyte lysate (100 μg of hemoglobin, lane 2), and testis (44 μg of protein, lane 3) separated on a SDS–polyacrylamide gel. (B) Immunoblot of the same gel as in A except for lane 1, 100 ng of purified aldose reductase being loaded and detected with ARAb. Arrowheads denote the migration of marker proteins (a, 69K; b, 46K; c, 30K).

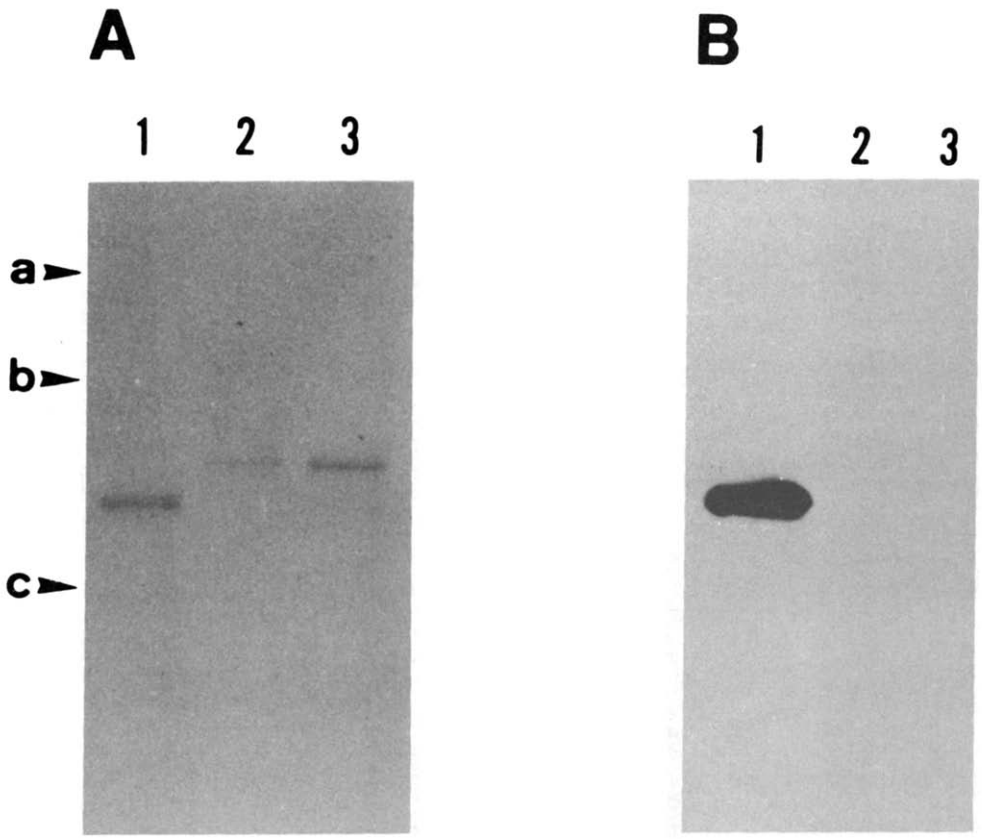

Fig. 3. Specificity of ARAb verified by immunoblot of purified preparations of aldose reductase and aldehyde reductase. (A) Coomassie Blue staining of purified recombinant aldose reductase (1 μg, lane 1) and aldehyde reductase isolated from human kidney (1 μg, lane 2; 2 μg, lane 3). (B) Immunoblot of purified aldose reductase (100 ng, lane 1) and aldehyde reductase (100 ng, lane 2; 200 ng, lane 3) detected with ARAb. Arrowheads denote the migration of marker proteins (a, 69K; b, 46K; c, 30K).

conditions (lanes 2 and 3). A couple of minor bands were detected in the testis preparation precipitated with preimmune IgG or ARAb (lanes 1 and 2). As shown in the immunoblot analysis (Fig. 1B); however, these protein species did not react with ARAb or CPAb. These minor bands thus appear to originate from nonspecific precipitation of testis protein with rabbit IgG. To validate this nonspecific reaction quantitatively, different amounts of immunoprecipitates were loaded on a SDS–gel and the staining intensity of the protein band was examined by densitometric analysis. The band near the 46 kDa marker (asterisk), the most intense nonspecific band, corresponded to no more than 4% of aldose reductase precipitated from the testis preparation (data not shown).

When the soluble fraction of human testis was directly subjected to the immunoblot analysis, ARAb reacted with a single band of protein identical in molecular size to the purified aldose reductase (Fig. 2; lanes 1 and 3). In the same blot, on the other hand, no reaction was detected in the erythrocyte lysate, indicating a lower level of aldose reductase expressed in human red blood cells (lane 2).

Many tissues contain, to a variable extent, both aldose reductase and aldehyde reductase with overlapping substrate specificity and partial amino acid sequence identity [23, 24]. To investigate the possible reactivity of ARAb with the purified human enzyme, aldehyde reductase was isolated from human kidney as described previously [25]. SDS–polyacrylamide gel electrophoresis demonstrated the homogeneity of this enzyme preparation (Fig. 3A). As shown in Fig. 3B, however, no cross-reaction of ARAb with purified aldehyde reductase was detected by immunoblot analysis. When 3–50 ng/mL of purified aldehyde reductase was applied to the current sandwich ELISA as an antigen for ARAb, only a background level (equivalent to 0 ng/mL) of O.D. reading was obtained up to 50 ng/mL of aldehyde reductase concentration (data not shown).

Quantitative determination of aldose reductase in human tissues by ELISA

As shown in Fig. 4, a linear standard curve was demonstrated up to 50 ng/mL of the purified enzyme concentration. Addition of known amounts of purified aldose reductase to the erythrocyte lysate of various dilutions resulted in a parallel increase in $O.D._{405}$. The recovery of the antigen at 160-fold

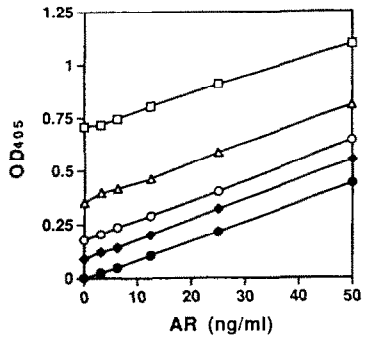

Fig. 4. Standard curve of sandwich ELISA and recovery of the antigen in the assay. Indicated amounts of purified aldose reductase (AR) were added to the hemolysate diluted 40- (□), 80- (△), 160- (○) and 320- (◆) fold. The standard curve (●) in the same assay is indicated. Similar results were obtained using two other blood samples.

Table 1. Aldose reductase in human tissues

Human tissue	Aldose reductase (μg/mg protein)
Kidney medulla	20.6
Sciatic nerve	3.8
Heart muscle	1.7
Testis	1.5
Cornea	1.4
Liver	0.8
Kidney cortex	0.7
Stomach mucosa	0.7
Spleen	0.7
Lung	0.5
Small intestine	0.4
Colon	0.4

The values represent the means of two to three separate assays determined by more than two serial dilutions in duplicate. The levels in heart muscle, cornea, liver and spleen are the average of two to three different tissues.

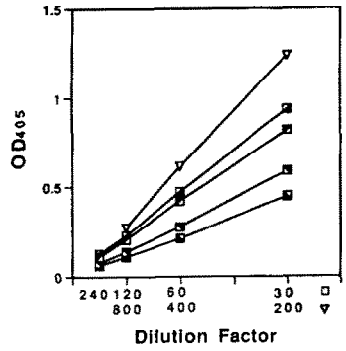

Fig. 5. Sample dilution curves in sandwich ELISA. Soluble fraction of the sciatic nerve (▽) and the hemolysate of four different blood samples (□) were serially diluted for incubation in ELISA.

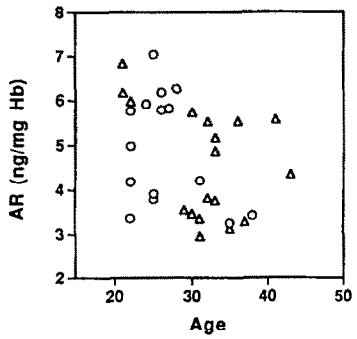

Fig. 6. The level of erythrocyte aldose reductase in healthy young adults. Each value (man: △; woman: ○) represents the mean of three to five separate assays determined by two serial dilutions in duplicate.

dilution of the hemolysate ranged from 98 to 107%. Next a serial dilution of the sample was incubated to assess the optimal dilution for determination of tissue aldose reductase in the ELISA system. As shown in Fig. 5 for the sciatic nerve and the blood samples, a linear response of immunoreaction was observed. When the intra-assay reproducibility was assessed with four separate blood samples at 60-fold dilution, the coefficient of variation (CV) ranged from 2.8 to 4.3%. On the other hand, the inter-assay reproducibility determined on three separate blood samples by 10–18 independent assays varied from 8.2 to 12.2% (data not shown).

The levels of aldose reductase expressed in various organs measured by ELISA are shown in Table 1. The highest level among organs investigated was found in kidney medulla, followed by sciatic nerve, heart muscle and testis. The values for testis (1.45 μg/mg protein) and kidney cortex (0.73) were in line with the results of the previous work, 1.8 and 0.83, respectively, determined by densitometric analysis of western blots using anti-bovine aldose reductase antibody [26]. The level determined for testis by the present ELISA also correlates well with our former study on testis aldose reductase [27].

The level of aldose reductase was measured in red blood cells of 33 healthy young adults, 18 men and 15 women ranging from 21 to 43 years of age. The values demonstrated more than a 2-fold variability in erythrocyte aldose reductase among these individuals (Fig. 6). The mean ± SD of the enzyme level determined in these normal subjects was 4.75 ± 1.21 (ng/mg Hb).

DISCUSSION

For the present immunoassay system, the recombinant aldose reductase expressed in a baculovirus system was utilized to raise antibodies and as a standard antigen, which is devoid of any other proteins of human origin. In our recombinant

enzyme preparation, the amount of coexisting insect cell protein was found to be negligible. This could be attributed to purifying enzyme protein recovered from the culture medium of the infected SF9 cells instead of from whole cell homogenate. Extracellular secretion of recombinant aldose reductase was observed irrespective of a variety of recombinant baculoviruses generated in our laboratory [12, 28]. This may be due to introduction of aldose reductase cDNA into baculovirus with a transfer vector pAcYM1 [29].

Comparison of the different antibodies isolated demonstrated that the highest sensitivity was obtained using anti-aldose reductase polyclonal antibody, ARAb. The antibody immunoprecipitated and reacted with the protein in the testis preparation identical to the molecular size of purified aldose reductase. Although amino acid sequence identity of approximately 50% between human aldose reductase and aldehyde reductase was reported [24], no cross-reaction of ARAb with tissue aldehyde reductase was detected. This indicates that ARAb invariably distinguishes between these two related enzymes and the antibody is specific for aldose reductase in human tissue.

An unexpectedly high level of aldose reductase was detected in the sciatic nerve among organs tested, aside from the kidney medulla where the osmoregulatory function of this enzyme was indicated [1]. In the previous immunoquantitative work based on western blot analysis [26], adrenal medulla of neural origin was also reported to contain high enzyme level. Considering the possible role of aldose reductase in the development of diabetic complications, the present finding may further endorse the significance of this enzyme in the pathogenesis of diabetic neuropathy.

Although the immunoblot analyses of our study and others [26] failed to detect the enzyme protein present in the erythrocyte, our immunoassay system was sufficiently sensitive for quantitative determination of the enzyme level in the hemolysate. When the validity of this system was examined on blood samples of healthy individuals, significant variation in aldose reductase level was demonstrated. This generally supported the previous findings on aldose reductase in the human placenta [9] and erythrocyte from diabetic patients [10] determined by a conventional enzyme activity assay method using isolated enzyme preparations. Quantitative determination of erythrocyte aldose reductase by the present ELISA provided direct evidence of the heterogeneity of this enzyme expression in a human population.

Since the efficacy of aldose reductase inhibitors can be associated with the variable level of tissue aldose reductase in diabetic patients, this ELISA system may be useful for determining the level of aldose reductase expressed in the target organs of diabetic complications. In this context, more work has to be done on the relationship between the level of aldose reductase in the erythrocyte and in other target tissues of diabetic complications. Also to be considered is the correlation of tissue enzyme level and enzyme activity, along with the accumulation of sorbitol under hyperglycemic conditions.

In conclusion, we have developed a new immunoassay system to enable direct measurement of aldose reductase protein in human tissues, circumventing isolation of this enzyme from other aldo-keto reductase family which co-exist in most of the human tissues. The sensitivity and specificity of this assay system may be useful for the evaluation of the effectiveness of aldose reductase inhibitors in diabetic individuals.

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