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Immunological detection of D-β-aspartate-containing protein in lens-derived cell lines

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Purpose: Although the presence of biologically uncommon D-β-aspartate (D-β-Asp) in lens protein is thought to be related to aging, we recently found this isomer in lens αA-crystallin from human newborns. The objective of this study was to examine whether D-β-Asp occurs in protein from lens-derived cell lines.
Methods: We examined the expression of D-β-Asp-containing protein in the lens-derived cell lines αTN4-1 and N/N1003A, by western blot and immunoprecipitation analysis using a polyclonal antibody against Gly-Leu-D-β-Asp-Ala-Thr-Gly-Leu-D-β-Asp-Ala-Thr-Gly-Leu-D-β- Asp-Ala-Thr (peptide 3R), which corresponds to three repeats of positions 149-153 in human αA-crystallin. The anti-peptide 3R antibody, prepared in a previous study, is a useful tool for investigating D-β-Asp-containing peptides.
Results: Western immunoblot and immunoprecipitation analysis showed that a 50 kDa protein in N/N1003A cells was strongly immunoreactive with the anti-peptide 3R antibody. Antibodies against αA- and αB-crystallin also stained this protein. On the other hand, the αTN4-1 cell line only expressed proteins of about 20 kDa, which also reacted to antibodies against αA-crystallin and αB-crystallin.
Conclusions: The results indicate that the N/N1003A cell line expressed a 50 kDa D-β-Asp-containing protein, which may share a common amino acid sequence with αA- and αB-crystallin.

Alpha-crystallin is the major protein of the mammalian lens and its average molecular weight is approximately 800 kDa. It is composed of two kinds of structurally and functionally related polypeptides, αA- and αB-crystallin subunits, each with a molecular weight of 20 kDa [1,2]. It has been reported that αB-crystallin is one of the small heat shock proteins [3]. We previously identified biologically uncommon D-β-isomers at Asp-58 and Asp-151 in αA-crystallin [4,5], and at Asp-36 and Asp-62 in αB-crystallin [6] from aged human lenses. D-β-Asp-151 of αA-crystallin was also found to be present in the lenses of cattle [7], horse [unpublished data], rat [8], and mouse [9]. D-β-isomers of Asp-58 in αA-crystallin and Asp-36 and Asp-62 in αB-crystallin were restricted to human lenses. These posttranslational modifications may cause the formation of cataract by affecting the close-packing of the crystallins and/or by reducing the chaperone-like activity of α-crystallin. Our previous study clearly revealed a large conformational change in the secondary structure of a peptide which corresponded to positions 146-157 of human αA-crystallin, caused by substitution of normal L-α-Asp by D-β-Asp-isomers [10].

Recently, we prepared a polyclonal antibody against peptide Gly-Leu-D-β-Asp-Ala-Thr-Gly-Leu-D-β-Asp-Ala-Thr-Gly-Leu-D-β- Asp-Ala-Thr (anti-peptide 3R antibody) that corresponded to three repeats of positions 149-153 in human αA-crystallin [11]. This antibody cross-reacted specifically with D-β-Asp-151-containing αA-crystallin. Because formation of D-Asp is accompanied by isomerization to form the β-Asp (isoaspartate) residue, three isomers of Asp residues, L-β-Asp, D-α-Asp and D-β-Asp isomers, are formed in the protein [12]. This antibody reacts only with the configuration of the D-β-Asp-containing peptide and not with the L-α-Asp, L-β-Asp and D-α-Asp-containing peptides [11]. Immunohistochemical staining of human lenses with this antibody demonstrated that D-β-Asp-151-containing αA-crystallin was predominantly localized in the core of aged human lenses [11]. In the lens, epithelial cells are induced to form differentiated lens fiber cells that constitute the bulk of the lens, where aged proteins accumulate because of lack of turnover. To date our research focused on aged lens tissues where protein may be irreversibly damaged and D-β-isomers at Asp-58 and Asp-151 in αA-crystallin were observed. However, a recent biochemical study showed that the formation of D-β-aspartic acid at Asp-151 of human αA-crystallin occurred even in newborn human lenses [12]. This finding suggests that D-β-aspartic acid formation may occur at an early stage of differentiation of the lens epithelium.

Cell culture systems are used widely for the analysis of cellular functions related to particular organ systems. For lens research, it is of particular interest to find conditions that reflect the situation within this organ. In order to establish whether the D-β-Asp-containing protein is present in cultured lens cells, we cultured two cell lines, αTN4-1 and N/N1003A, which are commonly used in lens research [13] and examined

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their protein expression by western blotting using anti-peptide 3R antibody. The results showed that D-β-Asp-containing protein was immunologically identified in one lens-derived cell line and further indicated that inversion and isomerization of Asp-residues in a protein may occur in actively proliferating cells.

METHODS

Cells: The αTN4-1 cell line was derived from a transgenic mouse carrying the promoter of the murine αA-crystallin gene and it was demonstrated by western blot analysis that all forms of α-crystallin were expressed [13,14]. This cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose and 10% fetal calf serum (FCS), which was heat-inactivated (56 °C, 30 min) before use. N/N1003A cells are rabbit cells known to express αB-crystallin [13,15]. These cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with rabbit serum (10%, not heat-inactivated). HH is a bovine carotid artery endothelial cell line [16], which was maintained in EMEM containing 10% FCS.

Antibodies: The following antibodies were used in this study: (a) anti-peptide 3R antibody, which was prepared in a previous study [11]. This antibody is a highly specific rabbit polyclonal antibody against Gly-Leu-D-β-Asp-Ala-Thr-Gly-Leu-D-β-Asp-Ala-Thr-Gly-Leu-D-β- Asp-Ala-Thr, which corresponds to three repeats of positions 149-153 in human αA-crystallin. The antibody clearly recognized the presence of D-β-Asp-containing αA-crystallin in aged human lenses [11]. We also synthesized four T18 variant peptides (IQTGLDATHAER, corresponding to the amino acid sequences 146-157 in human αA-crystallin) in which the Asp-151 residues were normal L-α-Asp, abnormal D-α-Asp, L-β-Asp and D-β-Asp, respectively. The 3R antibody can distinguish the configuration of the Asp-residue, that is, it reacts very strongly with the D-β-Asp-containing T18 peptide but not with the L-α-Asp, L-β-Asp and D-α-Asp-containing T18 peptides [11]; (b) two rabbit polyclonal antibodies against αA- and αB-crystallin (StressGen, Victoria, BC, Canada); (c) a goat polyclonal antibody raised against HSP27 (Santa Cruz Biotechnology, Santa Cruz, CA); (d) a goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP, Biosource International, Camarillo, CA); and (e) a donkey anti-goat IgG-HRP (Santa Cruz Biotechnology).

SDS-PAGE, Immunoprecipitation and western-blot analysis: Immunoprecipitation studies were carried out as follows; N/N1003A cells were seeded at approximately $1x10^6$ cells per 60 mm dish in culture medium and later harvested at 80-90% confluency using a cell scraper. The mediun was removed and the cells were washed with phosphate-buffered saline (PBS, pH 7.4), suspended in PBS on ice, then sonicated and centrifuged at 15,000x g for 10 min at 4 °C. To this supernatant (500 µl), 100 µl of protein A-sepharose beads (Amersham Pharmacia Biotech, Upssala, Sweden) were added and then incubated for 4 h at 4 °C. After the incubation, the mixture was centrifuged at 15,000x g for 1 min at 4 °C and the supernatant was collected and mixed with protein A sepharose beads which had been adsorbed by 10 µl normal rabbit serum (Dako Japan, Kyoto, Japan) to eliminate proteins which nonspecifically bind to rabbit immunoglobulin. The suspension was then centrifuged at 15,000x g for 1 min at 4 °C. To the resulting supernatant the protein A sepharose beads (which had been adsorbed by 10 µl anti peptide 3R antibody) were added and then incubated for 4 h at 4 °C. The beads were collected by centrifugation at 15,000x g for 1 min at 4 °C and then washed three times with 500 µl of washing buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40) and then suspended in 50 µl of Laemmli SDS sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 10% glycerol and 0.1% bromphenol blue) and heated at 90 °C for 5 min. Immunoprecipitated proteins were analyzed by SDS-PAGE (15%) and stained with Coomassie brilliant blue. Protein concentrations were measured with the BIO-RAD Protein Assay (Bio-Rad Laboratories, Hercules, CA). For western blot analysis, proteins were separated by SDS-PAGE and then transferred to Immobilon™ Transfer PVDF Membrane (0.45 µm, Millipore, Bedford, MA). The membrane was blocked with 5% non-fat milk in PBS overnight at 4 °C. The membrane was washed with PBS containing 0.1% Tween 20 and then incubated for 1 h at room temperature with the first antibodies (1:500). After four washes with PBS containing 0.1% Tween 20, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated second-

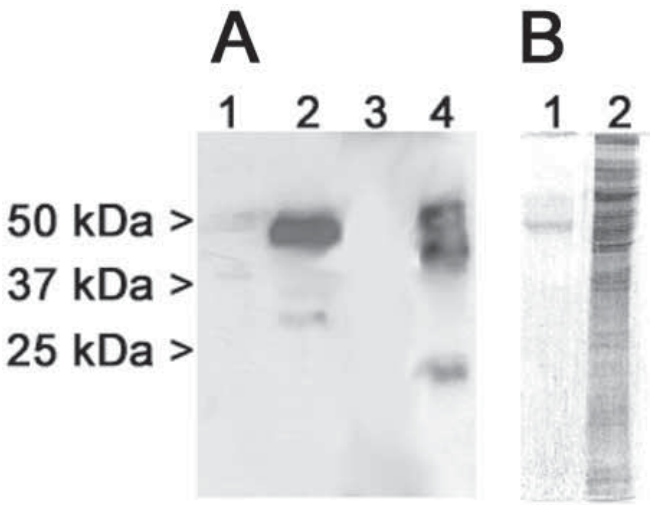

Figure 1. Detection of D-β-Asp-containing protein by western blot analysis using anti-peptide 3R antibody. SDS-PAGE (15%) and western blot analysis of protein (7 µg protein loaded) from the lens-derived cell lines were performed. **A**: Lane 1 contains protein from αTN4-1 cells, lane 2 contains protein from N/N1003A cells, lane 3 contains protein from HH cells, and lane 4 contains α-crystallin from the lenses of elderly donors. **B**: Immunoprecipitation of N/N1003A cell lysate with anti-peptide 3R antibody. Lysate of N/N1003A cells (containing about 0.2 mg total protein) was incubated with anti-peptide 3R antibody and immunoprecipitated protein was analyzed by SDS-PAGE. The gel was stained with Coomassie brilliant blue. Lane 1 contains immunoprecipitated protein and lane 2 contains total cell lysate.

ary antibodies (1:1000). Following another four washes with PBS containing 0.1% Tween 20, labeled proteins were visualized using enhanced chemiluminescence (ECL Western blotting detection kit, Amersham, Buckinghamshire, England) on BioMax MS film (Kodak, Rochestor, NY).

RESULTS

Expression of D-β-Asp-containing protein: To detect D-β-Asp-containing protein in the lens-derived cell lines, proteins were extracted from αTN4-1 and N/N1003A cells, and analyzed by western blotting using an antibody specific for D-β-Asp-containing protein/peptide (anti-peptide 3R antibody). Figure 1A shows that immunoreactive proteins were strongly expressed in the N/N1003A cell line (Figure 1A, lane 2), while little or no reaction was detected in αTN4-1 cells (Figure 1A, lane 1). The strongly-stained protein band had a molecular weight of about 50 kDa. This immunoreactivity was abolished by pre-addition of the D-β-Asp-containing peptide (Gly-Leu-D-β-Asp-Ala-Thr-Gly-Leu-D-β-Asp-Ala-Thr-Gly-Leu-D-β-Asp-Ala-Thr, data not shown). Proteins from HH cells were examined in parallel as a negative control (Figure 1A, lane 3). Since human α-crystallin extracted from 80-year-old lenses contained a large amount of D-β-Asp residues [4], we used this as a positive control (Figure 1A, lane 4). The two bands with higher molecular weight in lane 4 of Figure 1A might be aggregation products of α-crystallin because this sample is extracted from lenses of 80-year-old human donors. We also examined the expression of D-β-Asp-containing proteins in the N/N1003A cells, using immunoprecipitation analysis (Figure 1B). Anti-peptide 3R antibody precipitated one prominent protein at about 50 kDa from N/N1003A cell lysates (Figure 1B, lane 1).

Expression of αA- and αB-crystallin: We checked whether the two cell lines, αTN4-1 and N/N1003A, were positive for the expression of αA- and αB-crystallin. Each cell extract was electrophoresed on an SDS-PAGE (15%) and transferred to a membrane. Western blot analysis was performed using a rabbit anti-αA-crystallin antibody (Figure 2) or anti-αB-crystallin antibody (Figure 3). αTN4-1 cells contained both αA- and αB-crystallin 20 kDa polypeptides (Figure 2 and Figure 3, Lane 1). However, N/N1003A cells did not express an αA-crystallin 20 kDa band, but did express a 20 kDa αB-crystallin band (Figure 2 and Figure 3, Lane 2). One clear protein band corresponding to 50 kDa was also detected in N/N1003A cells with both anti-αA- and anti-αB-crystallin antibodies (Figure 2 and Figure 3, Lane 2). In HH cells, the 20 kDa protein band was detected by anti-αB-crystallin antibody (Figure 3, Lane 3) while no bands were observed using anti-αA-crystallin (Figure 2, Lane 3). These data suggested that the 20 kDa band in HH cells might be one of the small heat shock proteins, which could also be detected by anti-αB-crystallin antibody. Aged human α-crystallin used as the positive control was detected by either anti-αA- or anti-αB-crystallin (Figure 2 and Figure 3, Lane 4).

Characterization of D-β-Asp-containing protein in N/N1003A cells: We next examined whether the 50 kDa protein band detected with anti-peptide 3R, anti-αA- or anti-αB-crystallin antibodies represents the same protein. The 50 kDa protein in N/N1003A cell lysate was first immnoprecipitated with anti-peptide 3R antibody and then western blot analysis was performed to test any cross-reactivity of the protein with anti-αA- and anti-αB-crystallin antibody. Figure 4 clearly shows that the three different antibodies reacted with the same 50 kDa protein band.

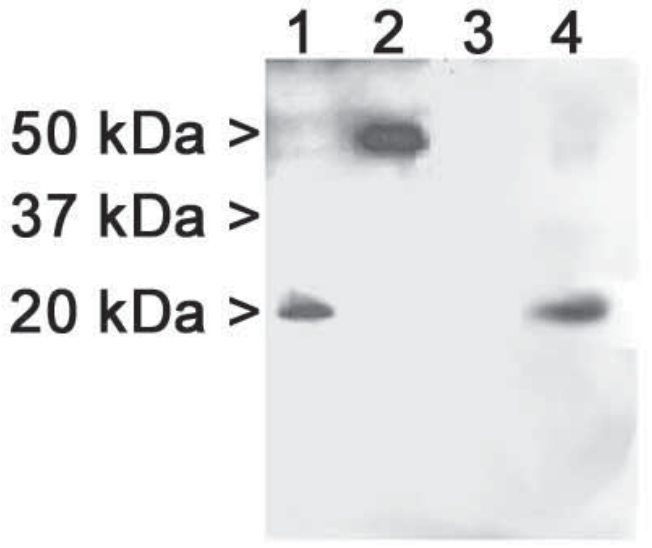

Figure 2. Detection of αA-crystallin in western blot analysis. Western blot analysis was performed with a rabbit anti-αA-crystallin antibody. Lane 1 contains protein from αTN4-1 cells, lane 2 contains protein from N/N1003A cells, lane 3 contains protein from HH cells, and lane 4 contains α-crystallin from the lenses of elderly donors.

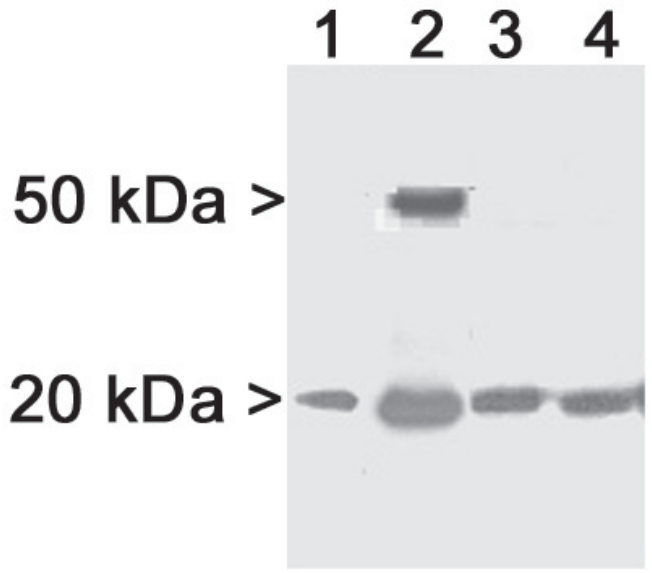

Figure 3. Detection of αB-crystallin in western blot analysis. Western blot analysis was performed with a rabbit anti-αB-crystallin antibody. Lane 1 contains protein from αTN4-1 cells, lane 2 contains protein from N/N1003A cells, lane 3 contains protein from HH cells, and lane 4 contains α-crystallin from the lenses of elderly donors.

Expression of HSP27: We further examined whether HSP27 was detectable in either cell line. Western blot analysis was performed with a goat polyclonal antibody raised against mouse HSP27 and a donkey anti-goat IgG-HRP antibody, respectively. HSP27 was not detected in either of the two cell lines (data not shown).

DISCUSSION

In previous studies, we reported that stereoinversion and isomerization occurred at Asp-58 and Asp-151 in αA-crystallin, and Asp-36 and Asp-62 in αB-crystallin from aged human lens tissues, forming isomers such as D-β-, D-α-, and L-β-Asp. The formation of these isomers started around birth, and the amounts increased during aging [12]. In many instances, cultured cell lines have been the system of choice to analyze cellular functions related to various organ systems. The purpose of the present study was therefore to determine if D-β-Asp-containing protein was present in lens-derived cells in culture. This study was successful in detecting immunologically the presence of D-β-Asp-containing protein in a lens-derived cell line. Western immunoblot and immunoprecipitation analysis showed that N/N1003A cells expressed a 50 kDa D-β-Asp-containing protein, whose identity remains unknown. Detection of human D-β-Asp-containing protein in lens-derived cells has, to our knowledge, not been reported previously. This 50 kDa protein was also stained by antibodies against αA- and αB-crystallins. The results suggested that the D-β-Asp-containing protein might share a common amino acid sequence with αA- and αB-crystallin. On the other hand, D-β-Asp-containing protein was not detected in αTN4-1 cells. This might be due to non-cross-reactivity of anti-peptide 3R antibody on this cell line of mouse origin by western blot analysis. Previous reports have revealed the relationship between αA- or αB-crystallin and small heat shock protein, but little is known about their roles. Therefore, we anticipated the expression of HSP27 in αTN4-1 and N/N1003A cell extracts but it was not detected in either cell line. αB-Crystallin, commonly accepted as a member of the heat shock protein family, was detected in all of the cell lines examined.

Mammalian lens cells in vivo synthesize α-, β-, and γ-crystallins. For the cell line αTN4-1, it was demonstrated by western blot analysis that all forms of α-crystallins are expressed [13]. The N/N1003A cell line has been widely used for studying α-crystallin [13,15,17]. Reddan, et al., described that N/N1003A cells expressed αA- and αB-crystallin polypeptides [15]; however, Krausz, et al., reported that they expressed αB-, but not αA-crystallin. [13]. Our data are consistent with previous reports [13,17]. Cultured lens epithelial cells, particularly cells from well-characterized strains, such as αTN4-1 and N/N1003A, should be useful for investigating the factors that regulate growth and crystallin gene expression. Since these cells retain lens-specific functions in long-term culture, they provide a stable system for investigating the regulation of crystallin gene expression [15].

The present report is the first to document the presence of D-β-Asp-containing protein in lens-derived cells, but further study is required to elucidate the identity of the 50 kDa protein and the physiological implications of this finding in the N/N1003A cell line. This cell line should be a powerful tool to study the mechanisms of D-Asp formation in protein and to search for possible enzyme activity for repair of unusual proteins.

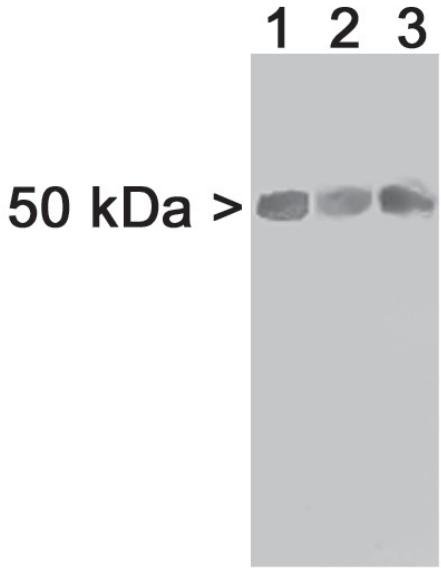

Figure 4. Analysis of immunoprecipitated protein. Analysis of immunoprecipitated protein with anti-peptide 3R antibody from N/N1003A cells by SDS-PAGE (15%) and western blot analysis. Lane 1 contains protein immunoprecipitated by anti-αA-crystallin antibody, lane 2 contains protein immunoprecipitated by anti-αB-crystallin antibody, and lane 3 contains protein immunoprecipitated by anti-peptide 3R antibody (positive control).

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