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Production and Immunocomparative Study of Human Lysosomal β-Mannosidase Antibodies

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Abstract.- Among glycosidases, β-mannosidase enzyme (EC 3.2.1.25) hydrolyze the non reducing end of N-linked mannose residue conjugated to glycoprotein and galacto(gluco)mannans. In mammals, an impaired activity of β-mannosidase leads to β-mannosidosis, In this study, antibodies against purified human lysosomal β-mannosidase were developed and characterized. The reactivity of β-mannosidase antibodies with cell free homogenates of human placenta, B and T cells and livers of mouse, goat and bovine was detected. Total human leukocytes were extracted on histopaque density gradient whereas human B and T lymphocytes were separated by panning method using anti-human CD22 and anti-human CD4/CD8 receptors antibodies, respectively. β-mannosidase enzyme was immunodetected in cell free homogenates by ELISA and slot blot analysis. The western blot analysis revealed the 97 KDa protein band in all homogenates of cell and tissues tested. Immunohistochemical studies indicated the deposition of reaction product on blood vessels, chorionic villi of human placenta and on the B and T cells. β-mannosidase antibodies will be effective tool to detect the β-mannosidase activity during β-mannosidosis and other clinically associated diseases.

Key words: Antibodies, β-mannosidase, immunohistochemistry, western blot.

INTRODUCTION

Many lysosomal glycosidases are important in the degradation of glycoproteins in normal cellular metabolism. Among them, lysosomal β-mannosidase enzyme (E.C. 3.2.1.25) digest the non reducing end of the β-linked mannose residue (β-1-4 GlcNac) in the degradation pathway of glycoproteins (Ademark *et al*., 1999; Jones *et al*., 1992; Sopher *et al*., 1993; Percheron *et al*., 1992). Deficiency or impaired activity of β-mannosidase in mammals leads to the excretion or accumulation of undegraded β-1-4 linked di and trisaccharides (mannosyl-N-acetylglucosamine) (Tjoa *et al*., 1990). Lack of β-mannosidase activity results in β-mannosodisis (lysosomal storage disease), an autosomal recessive inherited disorder. β-mannosidase activity has been characterized in many species such as fungus (Ademark *et al*., 1999; Kurakake, 2001), hyperthermophilic microbes (Duffaud *et al*., 1997; Bauer *et al*., 1996), plant (Mo and Bewley, 2002), goat (Pearce *et al*., 1990), bovine (Sopher *et al*., 1993) and human (Guadalupi *et al*., 1996).

β-mannosidosis, a genetic disease was detected in goat (Jones *et al*., 1983), bovine (Jolly *et al*., 1990) and has also been characterized in human (Kleijer *et al*., 1990; Van *et al*.,1990; Alkhyat *et al*., 1998). The investigation of the clinical / pathological features of Nubain goat offspring (Render *et al*., 1988) and salers calves (Abbitt *et al*., 1991) suffered from β-mannosidosis which is documented as facial dysmorphism, dom shaped skulls, hyper extensuion of joints, muscle atrophy, marked intention trimor, inability to stand, deafness, myelin carporal contracturs (Render *et al*., 1988; Bryan *et al*., 1990), cytoplasmic vacuolation, accumulation of oligosaccharides in fibroblast, leukocytes, brain , kidney, liver and demyelination in central nervous system (Lovell *et al*., 1994; Patterson *et al*., 1991; Uchino *et al*., 2003).

In human, total 18 cases and thirteen families with β-mannosidosis have been reported (Gort *et al*., 2006). β-mannosidosis in human is considered as milder clinical expression conditions with heterogenecity (Bedilu *et al*., 2002). Clinical studies of β-mannosidosis in humans documented mild peripheral neuropathy (Levade *et al*., 1994), hearing loss and speech impairment (Poenaru *et al*., 1992) epileptic encephalopathy (Cooper *et al*., 1991), angiokeratome (Rodriguez-Serna *et al*., 1996), facial dysmorphism (Kleijer *et al*., 1990) reduced β-

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mannosidase activity in fibroblast, plasma and leukocytes (Cooper *et al*., 1988) and accumulation of disaccharides (Van *et al*., 1990). DNA sequence of β-mannosidase and mutation associated with β-mannosidosis has been identified (Alkhyat *et al*., 1996; Sedel *et al*., 2006).

In order to characterize the expression level and activity of β-mannosidase in human tissues and immunocomparison with other mammalian species (mouse, goat and bovine), antibodies were developed against human β-mannosidase and used for immunodetection of β-mannosidase activity in species as mentioned above. Here, we report first time β-mannosidase activity in B and T cells and histochemical finding in placental tissue.

MATERIALS AND METHODS

The chemicals and materials required for experimentation and buffer preparation were of analytical grade and purchased from Sigma-Aldrich, USA.

Denaturation of β-mannosidase

Purified human β-mannosidase (β-MAN) (1mg/ml) was mixed with denaturing buffer (50mM Tris-Cl, pH 7.4, 0.01% SDS, 0.005M β-mercaptoethanol) in 1:1 ratio and kept at 60^{o}C for 30 minutes to denature the β-mannosidase to avoid the self tolerance of mammalian antigen.

Production and partial purification of antibodies

Two male Balb/C mice (weighing 200-250gm), 6-7 weeks old were injected subcutaneously with denatured β-mannosidase (30-40 µg per injection) after mixing with Freunds complete adjuvants in 1:1 ratio at two weeks intervals with a total of five injections. In order to check the antibody titre, 100 µl blood from tail vein or from orbital vein was collected as described (Talwar, 1983). Serum was isolated and checked for reactivity by indirect Enzyme linked immunosrbant assay (ELISA) using denatured human β-mannosidase. After getting sufficient titre, the mice were anesthetized by chloroform and whole blood was isolated by cardiac puncture. An aliquote of serum was stored at -20^{o}C. For partial purification, serum was mixed with $(NH_4)_2SO_4$ at 50% saturation. The precipitated proteins were centrifuged at 5000 rpm for 10 minutes at 4^{o}C. The pellet was dissolved in 0.05 M Tris-Cl, pH 7.4 and dialyzed against 2 litres of same buffer. The dialyzed antibodies were stored at –20^{o}C for further analysis. Preimmune control serum was obtained from mice tail before immunization.

Extraction of Human leukocytes and lymphocytes

Human peripheral blood leukocytes were isolated from 10 ml hepaninized blood as described in Johnstone and Thrope (1987). Briefly, normal blood samples were mixed with balanced salt solution (BSS) (0.0007 % $CaCl_2$, 10% glucose, 0.02% $MgCl_2$, 0.04% KCl and 0.15M Tris-Cl pH 7.6) in1:1 ratio. Diluted blood samples were loaded on 30 ml Histopaque-Ficoll density gradient in 50 ml sterilized falcon tube and were centrifuged at 2000 rpm for 25 minutes at 4^{o}C. White buffy coat containing leukocytes were isolated and washed three times in 10 ml BSS by centrifuging at 800 rpm for 10 minutes at 4^{o}C. Total cell population was calculated by trypan blue exclusion test (Johnstone and Thrope, 1987) and the concentration of the cells was adjusted to 1 x 10 ^{8} cells / ml.

Normal B and T-lympotocytes were extracted from buffy coat by panning method using their cluster of differentiation marker (CD) using basic procedure of Johnstone and Thrope (1987) and Wysocki and Sato (1978) with modification. 20µl (1mg/ml) of reconstituted anti-human CD22 receptor (for B-lymphocytes) and anti-human CD4 and anti-human CD8 receptors (for T-lymphocyte population) monoclonal antibodies were mixed with 25 ml BSS and added in 90 mm plastic petri plates separately. The plates were kept at 37^{o}C for 30 to 35 minutes and the supernatant was decanted and saved for reuse. Extracted buffy coat as given above was suspended in 15 ml BSS and added into plastic plates coated with antibodies and kept at 37^{o}C for 25 to 30 minutes under gentle shaking. The adhered cells were observed under microscope and detached by triturating the BSS 5-6 times. Triturated cells were centrifuged at 1000 rpm for 10 minutes at 4^{o}C. Cells were collected and calculated by trypan blue dye exclusion test. The cell concentration was adjusted to 1 x 10^{8} cells /ml.

Extraction of cell free mammalian proteins

10 g of human placenta tissue and liver of normal mouse (Balb/C), goat and bovine each separately were cut into small pieces and washed thoroughly with sterilized washing buffer (10mM Tris-Cl, pH 7.4). These tissue samples were mixed separately with 2 ml homogenization buffer (0.1M acetate buffer, pH 5.0 including protease inhibitors with final concentration, 1mg/ml aprotinin, 1mg/ml leupeptin, 1mM EDTA, 1mM phenylmethyl-sulfonylfluoride) in homogenizer on ice and minced. Minced sample were centrifuged at 10,000rpm for 10 minutes at 4^{o}C. Supernatants were isolated and the protein concentration was measured with bradford dye binding assay using bovine serum albumin as standard (Bradford, 1976). The protein concentration was adjusted to 1mg/ml and stored at –20^{o}C.

The protein contents of leukocytes, B and T cells were extracted by homogenizing the cells in homogenizing buffer as described above. The protein contents were adjusted to 1mg / ml.

The cell free homogenates of leukocytes, B cells, T cells and livers of mouse, goat and bovine were mixed with denaturing buffer to destroy the endogenous phosphatase activity.

Enzyme activity

The activity of β-mannosidase in cell free homogenates of human placenta, leukocytes, B cells, T cells and liver of mouse, goat, bovine was detected using p-nitrophenyl-β-D-mannopyranoside as substrate (Uchino et al., 2003). Reaction mixture contains the 300 µl of 2mM p-nitrophenyl-β-D-mannopyranoside and 20µl of extracted proteins in 0.1M acetate buffer pH 5.0 and incubated at 37^{o}C for 3 hours. The reaction was stopped by adding 1.65ml of 0.25 M NaOH and free p-nitrophenol was measured spectrophotometrically at 410nm. One unit of enzyme is defined as the amount of enzyme which releases of 1.0µmol of *p*-nitrophenol per minutes at 37^{o}C.

Enzyme linked immunosorbant assay

10µl of purified denatured human β-mannosidase (1mg/ml), 10 µl of extracted denatured supernatant from placenta, leukocytes, B cells, T cells and liver of mouse, goat, bovine were mixed with 200µl of 50 mM carbonate buffer, pH 9 and was absorbed on microtitre ELISA plates for overnight at 4^{o}C. The nonspecific binding sites were blocked with blocking buffer (3% bovine serum albumin in Tris-buffered saline-Tween 20, (TBST) for an hour at 37^{o}C with continuous shaking. After washings in TBST, the partially purified mouse anti-human β-mannosidase antibody (1:500 dilution) was added in wells and kept at 37^{o}C for an hour with continuous shaking. The plates were again washed in TBST. After washing, alkaline phosphatase (AP)-conjugated rabbit anti-mouse IgG antibody (1:5000 dilution) was added in wells and incubated at 37^{o}C for 30 minutes. After washings in TBST, colour reaction was developed using para-nitophenyl phosphate (PNPP) as a substrate (Harlow and Lane, 1988). Control assays included pre-immune serum and secondary antibody alone.

Immunoslot blot analysis

20µl of purified denatured human β-mannosidase (1mg/ml) and 25µl of extracted supernatant from placenta, leukocytes, B cells, T cells and livers of mouse, goat and bovine each were mixed separately with 500µl denaturing buffer. The samples were loaded in the Hybri slotblot apparatus (Bethesda Research Laboratories, USA) and followed the instructions given by manufacturer. After absorbing the proteins samples, the nitrocellulose membranes were washed with TBST and stained with acidic ponceau S stain to confirm the absorption of proteins. After destaining, the membranes were treated with blocking buffer (3% BSA in TBST) and then processed further for immunochemical reaction as described in ELISA section above. The colour reaction was developed using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoloyl phosphate (BCIP) as substrates. Control assays were done with preimmune serum and secondary antibody (Harlow and Lane, 1988).

SDS-PAGE and western blotting

The monospecificity of the antibodies was checked by western blot analysis as described (Towbin *et al.*, 1979). Electrophoresed proteins samples on 10% SDS-polyacrylamide gel (Laemmli,

1970) were transfered on to nitrocellulose membrane. After blocking the non specific binding sites on nitrocellulose paper with blocking buffer (3% BSA in TBST), blots were incubated with primary antibodies (mouse anti-β-mannosidase antibodies) and secondary antibody (AP-conjugated rabbit anti-mouse IgG) as described in ELISA. After five washing in TBST, colour reaction was developed using NBT and BCIP as substrate. Control assays were performed with pre-immune serum and secondary antibody.

Immunohistochemistry and light microscopy

Human placental tissues were cut into small pieces, washed in washing buffer (50 mM Tris-Cl, pH 7.4) and fixed in PLP fixative (Paraformaldehye-Lysine-Periodate) (Samra *et al*.,1990; McLean and Nakane, 1974). Briefly, fixed tissues were dehydrated in ascending series of ethanol (50% to 100%) and infiltrated with xylene and paraffin (5% in xylene) at 50^{o}C and were embedded in paraffin block. Sections (8-10 µm) of tissues were cut in microtome and placed on egg albumin coated slides and processed for immunolabelling.

Paraffin sections after fixing on slides were deparaffinized by running the slides through xylene and then descending series of ethanol (100% to 50%) and finally in TBS. The rehydration tissues were treated with 0.5% H_2O_2 solution to block the endogenous peroxidase activity. After washing and treating with blocking solution (3% BSA / TBS), mouse anti-β-mannosidase antibodies (1:400 dilution) were added onto the slides in humidified chamber for 45 minutes. After washing, peroxidase conjugated goat anti-mouse IgG (1:2000 dilution) was added onto slides for 30 minutes. After washing in TBST, samples were incubated with 1% glutaraldehyde for 5 minutes followd by treatment with 0.02 % diaminobenzidine in 0.05 M Tris-Cl, pH 7.4 for 30 minutes and then transfred to the same medium containing 0.5 % H_2O_2 for 5-10 minutes. A 5-10µl glycerol solution was added on the slides and observed under the microscope. Control slides were treated with pre-immune serum and secondary antibody alone.

B and T cells (1 X 10^4) were added separately on albumin coated slides and mixed with 50µl of 1% glutaraldehyde in TBS to fix the cells on slides. After gentle washing, the slides were immersed in 0.05% H_2O_2 in TBS for 2 to3 minutes to block the endogenous peroxidase, activity. The slides were rinsed with TBS and processed for immunolabelling as described above.

Morphological studies

Slides containing the 8-10µm sections were deparaffinized by incubating the slides in xylene for 15 minutes. The deparaffinized slides were rehydrated in descending series of ethanol (100% to 50%) and finally in TBS. The slides were incubated in hematoxyline for 2 minutes and then exposed to eosin solution for 2 seconds. After washing in 100% ethanol, glycerol was added on the slides and observed under microscope.

RESULTS AND DISCUSSION

The β-mannosidase is an important enzyme to regulate the mannose-glycoprotein metabolism in lysosome. The defect in β-mannosidase gene expression leads to lysosomal storage disease, known as β-mannosidosis (Pearce *et al*., 1990; Sopher *et al*., 1993, Guadalupi *et al*., 1996, Lovell *et al*., 1994; Patterson *et al*., 1991; Uchino *et al*., 2003). The development of anti-human β-mannosidase antibodies and its immunochemical characterization will be helpful to elucidate the expression level of β-mannosidase in normal as well as in the individuals suffering from β-mannosidosis.

Antibodies were developed against purified denatured β-mannosidase for detecting the specific reactivity in other mammalian tissue samples. After characterization of antibody titre in ELISA, the specifity and reactivity of β-mannosidase antibodies was checked under denaturing conditions. Western blot analysis of purified β-mannosidase confirms the reactivity of antibodies (Fig.1).

An immunoslot blot analysis of homogenates of different tissue samples revealed the reactivity of anti-β-mannosidase antibody against the mammalian denatured β-mannosidase (Fig.2). In order to analyze the immunospecificity of antibodies, western blot analysis of homogenates from placenta, leukocytes, B and T cells and liver of mouse, goat and bovine were processed (Fig.3) and

the deposition on 97 KDa protein bands in all samples confirmed the common antigenic sites in β-mannosidase enzyme among mammalian species.

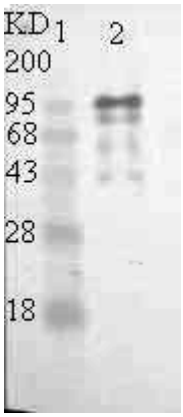

Fig. 1. Western blot analysis of purified human β-mannosidase. Partial degradation of β-mannosidase enzyme was observed on storage at -20°C.

Fig. 2. Immunoslot blot analysis. Reactivity of anti human β-mannosidase antibodies with homogenate extract of, (1), human placenta (2), human leukocytes (3), human B cells (4), human T cells (5), goat liver (6), bovine liver (7), Balb/C mouse liver and (8), purified β-mannosidase. The reactivity indicated the common epitopes in β-mannosidase.

When paraffin sections of human placenta were examined by negative staining, good morphological preservation in placental tissues was seen (Fig. 4). The morphological structure preservation of paraffin cut section of placental tissue was good and no disruption of tissue was seen. The structural studies of the cut sections were compared with reported placental tissue as mentioned in Reith and Ross (1977). The paraffin sections of human placenta examined by immunoperoxidase microscopy using antibodies to human β-mannosidase, diaminobenzedine reaction product was decorated over blood vessel, chronic villi, itravillous space and cytoplasmic granules (Fig. 5). The B and T cells were also decorated with brown reaction product (Fig. 6) confirming the presence of β-mannosidase activity.

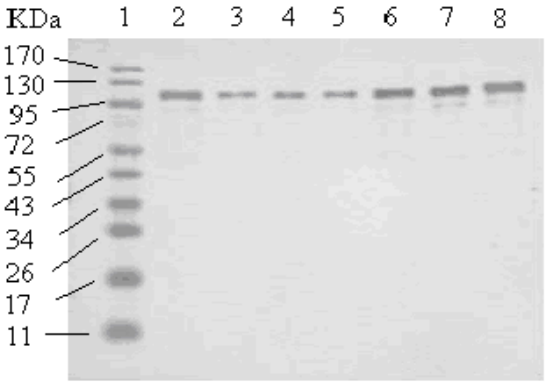

Fig. 3. Western blot analysis of tissue homogenates. The reactivity of anti-human β-mannosidase antibodies is at 97KDa protein band. Lane1, molecular weight marker (KDa), Lane2, homogenate of human placenta, Lane3, homogenate of human leukocytes, Lane4, homogenate of human B cells, Lane5, homogenate of human T cells, Lane6, homogenate of goat liver, Lane7, homogenate of bovine liver, Lane8, homogenate of Balb/C mouse liver.

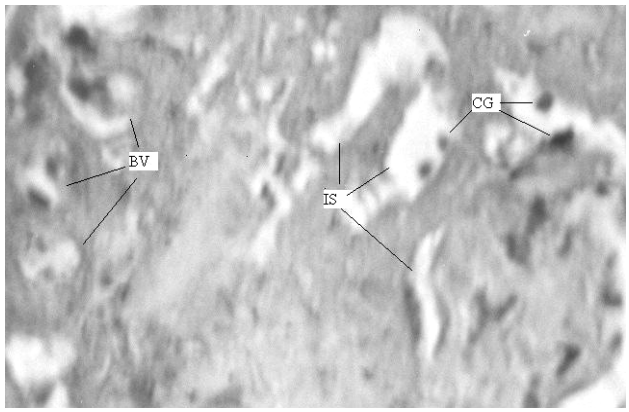

Fig. 4. Negative staining on paraffin section of human placenta. Morphology is

visualized under light microscope, 200x magnification. BV (blood vessel), IS (intervillous space), CG (cytoplasmic granules) are seen.

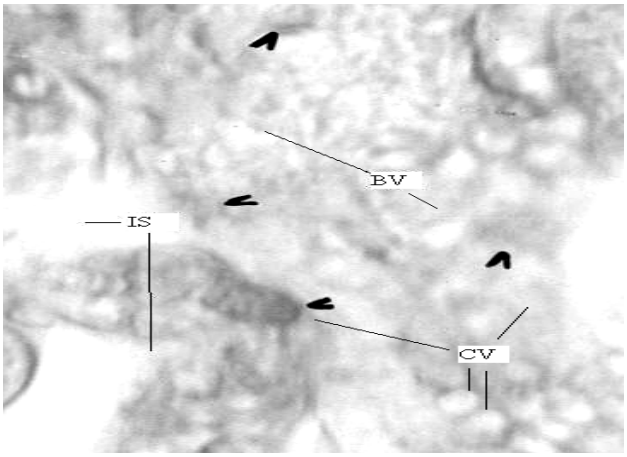

Fig. 5. Indirect immunoperoxidase microscopy of paraffin section of human placenta incubated with mouse anti-human β-MAN antibodies, 200x magnification. Reaction product is deposited on chorionic villi (CV) (arrow head). IS (Intervillous space), BV (Blood vessel).

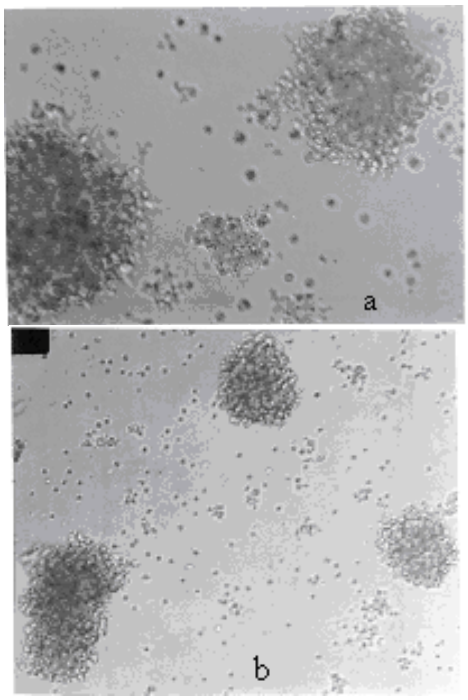

Fig. 6 Indirect immunoperoxidase labeling of B and T cells at light microscopic level using anti-human β-MAN antibodies, 500x magnification. (a), B cells and (b) T cells showed the immunoreactivity which indicate the presence of β-mannosidase activity in the lymphocytes.

Our immunochemical data indicates that β-mannosidase enzyme is present in human placenta, leukocytes, B cells, T cells. The human β-mannosidase antibodies also showed reactivity with other mammalian β-mannosidase. The antibodies may provide fruitful insight in the β-mannosidosis patients as well as in recombinant animal model system.

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