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Co-Deposition of Serum Amyloid P Component in mouse Brain following Chronic Inflammatory Mice

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Abstract: *Background: Prolonged injection of casein results in systemic amyloidosis in mice with impaired hepatic functions due to excessive serum amyloid P component (SAP) deposition in liver, spleen, kidney and brain.*

Methods: We have carried out radiolabeling, fluorescence immuno staining and brain endothelial immune staining studies to source the serum amyloid P component (SAP) that present in the brain of the affected mice.

Results: The experiments suggest that the systemic amyloidosis results in selective crossing of SAP proteins through blood brain barrier (BBB). Injection of ^{125}I SAP to casein treated mice resulted in the accumulation of this proteins in the liver to the maximum extent and to about 20% (compared to liver) localization in the brain. Immuno histochemical studies also indicate that the abundance of SAP in the mice brain following chronic inflammatory condition.

Conclusion: The present work provides a basis for investigation on the effect of chronic inflammation to the

Keywords: Systemic amyloidosis, serum amyloid A, serum amyloid P component, blood brain barrier, and radiolabeling.

1. Introduction

Serum amyloid P component (SAP), a member of highly conserved lectin fold superfamily and pentraxin serum protein family, consists of fi ve noncovalently associated identical subunitsSerum Amyloid A (SAA) and serum amyloid P component (SAP) is an acute phase reactant, whose level in the blood is elevated to 1000 fold when the body is subjected to various metabolic insults like trauma, infection, inflammation, and neoplasia (Kushner, 1982) . Serum amyloid P component (SAP) is found to be up-regulated in amyloidotic condition. It is a glycoprotein composed of subunit of approximately 25 kDa that has been found in association with almost all types of amyloid deposits. It is suggested that by controlling SAP production at the tissue level, the inflammatory damage in the brain due to Alzheimer's disease (AD) may be controlled. The present work aims explore the possibility of SAP crossing the BBB and getting accumulated in the brain in systemic amyloidotic condition. For the first time we have shown that in chronic amyloidotic conditions, d SAP is selectively transported to the brain, which resulted in SAP accumulation in the brain. Regardless of the protein precursor of amyloid fibrils in the different clinical types of systemic amyloidosis (Richards et al, 2015), all amyloid deposits also always contain abundant SAP, an invariant, normal, nonfibrillar plasma glycoprotein. SAP binds avidly but reversibly to all types of amyloid fibrils and is thus specifically concentrated in all amyloid deposits (Pepys and Hawkins, 2010). Native SAP binds to DNA, chromatin, glycosaminoglycans, amyloid fi brils, bacteria, bacterial lipopolysaccharide (LPS), complement components, and polymorphonuclear neutrophil cells. The physiological roles of SAP include among others the clearance of cellular debris at the sites of infl ammation and protection against chromatin-induced autoimmunity (Pepys et al, 2012 and Veszelka et al, 2013). Accumulation of amyloid fibrils in the viscera and connective tissues causes systemic amyloidosis, which is responsible for about one per thousand deaths in developed countries. Serum amyloid P component (SAP) is selectively concentrated in amyloid deposits by its avid binding to all amyloid fibril types. SAP binding stabilises amyloid fibrils, protects them from proteolysis in vitro5 and contributes to pathogenesis of systemic amyloidosis *in-vivo* (Bodin et al, 2010).

2.0. Materials and Methods

2.1. Production of Systemic Amyloidosis

Colony-bred adult male Swiss White mice (30-35 g) were used. Animals were selected randomly and caged in groups of four at room temperature (25-35° C) and supplied with food (Commercial pelleted animal feed marketed by M/s. Hindustan Lever, Bombay, India under the name "Gold Mohur rat feed") and tap water ad libitum (control n=5, test n=5). All animal procedures were carried out as approved by the Animal Care and Use Committee of Central Leather Research Institute, Chennai. Mice were given 0.5 ml of 10% Vitamin free Casein (1CN Pharmaceuticals, Cleveland, OH, USA) as subcutaneous injection (Botto et al., 1997) for 66 days to induce chronic inflammation of systemic amyloidosis. Control animals were treated with saline. At the end of treatment, animals were decapitated. Immediately after decapitation, brain cerebral cortex, liver, and spleen were removed in the ice-chilled condition for further analysis. Systemic amyloidosis was confirmed by "ladder formation" in the liver. The presence of

amyloid fibrils in the liver and Spleen is further confirmed by Congo red staining.

2.2. Immunostaining of Brain Endothelial Cell Capillaries

Brain capillary endothelial cells were prepared as described previously (Saikotos, 1974). Briefly, six fresh Swiss White mice brains were homogenized with 0.4M sucrose containing, 0.1 mM $CaCl_2$, 1mM ATP, and 0.5mM MES buffer pH 6.1. The homogenate were filtered using 50-60 size nylon meshes. The filtrate was centrifuged at 7000g for 45 min. The resulting supernatant was passed through the 1.1 × 40 cm Sephadex G-25 columns. Final purification was achieved by foam fractionation in a media of high surface-active substance, such as bovine serum albumin. The endothelial cells were identified by their characteristic morphology of extremely attenuated flattened shapes with nuclei lining the capillaries. The capillaries are viewed with in the inverted phase-contrast microscopy (Olympus CK30-F200, Japan). After fixing the portion of the brain endothelial cells over the glass slides, the immuno labeling were carried out by following procedure. Rabbit polyclonal antibody to mice SAP, which recognize the SAP, was used for immunolabeling. The whole serum was diluted 1:1000 in 0.1M phosphate buffer saline, pH 7.4. After blocking the nonspecific binding with 2% BSA solutions, the section were incubated for 30 min the room temperature in SAP primary antibody. Controls were incubated with non-immune rabbit serum (diluted with 1:1000) and with anti SAP antibody. After washing the sections were treated with FITC conjugated secondary antibody (Sigma, USA). Fluorescence in the slides was observed Zeiss fluorescence microscopy (AXIO plan2, Carl ZEISS NAG f. HBO50, Germany).

2.3. Isolation of SAP

SAP was isolated from plasma of casein-injected mice. The pooled plasma was processed for SAP extraction as previously described (Pepys, 1979). 12% SDS-Polyacrylamide gels characterized SAP. The isolated SAP was purified by Waters RP-HPLC analytical column 4.6 × 250 mm Spherisorb ODS2 LC_{18} (Waters, Milford, Massachusetts, USA) and the molecular weight determined by SEC columns used to 7.8 × 300 mm Ultrahydrogel 250™, Ultrahydrogel 500™ (Waters, Kyoto, Japan.) column series (Kaplan et al., 1999).

2.6. Immunostaining of Brain Sections

The antisera against SAP were prepared as per the following procedure. Briefly, New Zealand White rabbits were given intradermal injection of 0.1 mg/ml of mouse SAP was given weekly once; prior to the injection, the SAP protein was dissolved in PBS and emulsified in complete and incomplete adjuvant. Three weeks later a fourth intradermal injection was given and the animal were bled in the following week. The antisera were stored for further processing in -20°C. By following procedure the immunostaining of SAP in the brain is carried out as follows; after blocking the nonspecific binding with 2% BSA solutions, after brain tissue sections were incubated with anti SAP for 30 min. at 4°C. After washing in PBS, the sections were incubated with FITC conjugated rabbit anti mouse antibody for 30 min. at 4°C.

2.7. Radio Labeling

The SAP was labelled with ^{125}I (Baba Atomic Research Center, New Mumbai, India) using the method by Hamazaki (Hamazaki 1995) and stored in phosphate buffered saline containing 0.02% NaN_3. Labeled proteins to a final specific activity of approximately 2mci/mM were given to amyliodotic and control mice. ^{125}I BSA used as a control. After 24 hrs, animals were sacrificed and the organs (brain, liver, and spleen) and plasma were measured in a 1270 Rack Gamma II γ counter (Pharmacia Biotech (LKB), Uppsala, Sweden). All the groups compared statistically using the Chi-spuare test, U-Test (Mann-Whitney-Wilcoxon) and analysis of variance (ANOVA) with 2-tailed significance thresholds.

3.0. Results

3.1. Transport of SAP across the Blood Brain Barrier

Suki et al (Suki, et al., 2000) have previously reported an enhanced accumulation of SAA protein in the brain of hypertensive monkey. Sourcing of this type of amyloid accumulation can be either due to local production or uptake from the blood. Considering the abundance of SAP expression in the peripheral tissues and enhanced the levels of SAP in the blood, the transport of SAP to brain tissues through the blood brain barrier is not totally ruled out. To find out whether SAP could pass the BBB, we injected the radio labeled ^{125}I SAP in the control and casein injected mice (mice with chronic inflammation). Fig. 1a indicates that the radiolabeled SAP enters into the brain of the mice affected by systemic amyloidosis. The SAP incorporation to the brain tissue is specific to the SAP in the amyloidotic condition, because the amyloidotic mice when injected with radio labeled ^{125}I BSA shows negligible accumulation of ^{125}I BSA in the brain. This observation precludes the possibility of leaky brain endothelial cell capillaries, which will allow most of the proteins to be diffused in the chronic inflammatory conditions.

Further, radio labeled ^{125}I SAP and ^{125}I BSA to the control shows no significant incorporation in the brain (Fig. 1a). The radio labeling analysis of the SAP is also carried out in plasma, liver, and spleen. Most of the SAP injected got accumulated in liver both control and amyloidotic mice (Fig. 1b). However in the spleen there is decreased accumulation when compared to that of liver (Fig. 1c). It should be mentioned that in control mice also got the SAP protein accumulated in the liver and spleen to the same extend. This may be due to general ability of SAP retention in these tissues, which explain their propensity to get accumulated amyloid deposits. The SAP level in the plasma was also higher than in the spleen, suggesting the clearance of SAP excretion is low (Fig. 1d). However the total clearance in the ^{125}I BSA in plasma, liver and spleen indicate

the protein is totally excreted within 24hrs from time of the injection. Even though the amount of SAP incorporation to the brain is not very high as in the case of plasma, liver, and spleen, there is still a significant accumulation found in the brain in the chronic inflammatory mice when compared to the control.

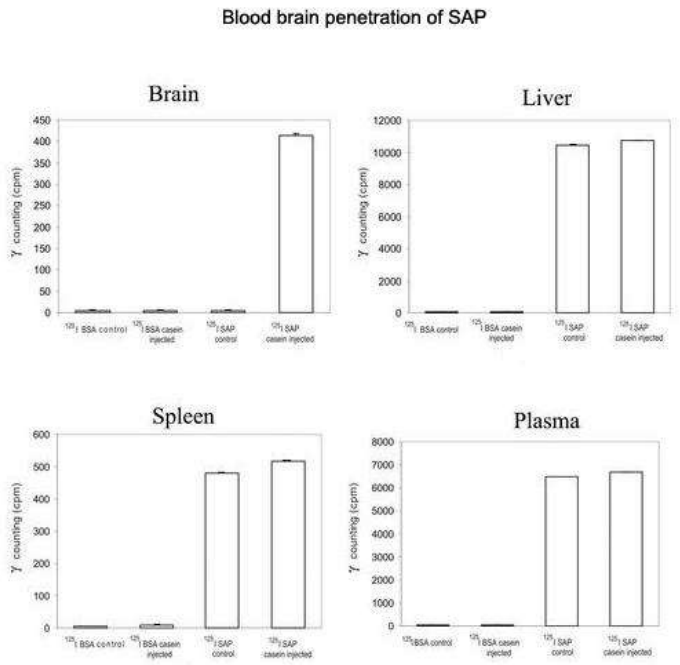

Fig.1. Incorporation of ^{125} I labelled SAP and ^{125}I Labelled BSA in the control and Casein injected mice (a) Accumulation of ^{125} I SAP in the Casein treated mice brain. Note less significant incorporation observed in control and BSA treated mice. (b), (c), and (d) shows correspondingly liver, spleen, and plasma of SAP. Most of the SAP accumulated in both control and Casein injected mice in various organs. However no significant in BSA incorporation in both control and Casein injected mice. Data show mean γ counting values (±SEM) from single, representative experiments. * $p < 0.05$; * * $p < 0.01$: * * * $p < 0.001$, related to control conditions.

3.2. SAP binding to the Brain Endothelial Cell Capillaries

Either the transport or accumulation of and SAP to the brain have to be through the endothelial cell binding/expression. The Immunostaining of brain endothelial cell capillaries shows the presence of and SAP in the endothelial cells of mice with chronic inflammation with antibody raised against SAP. The fluorescence microscopic picture shows that SAP selectively binds to the brain endothelial cell capillaries (Fig. 2a). In Fig. 2b control and endothelial capillaries show no staining.

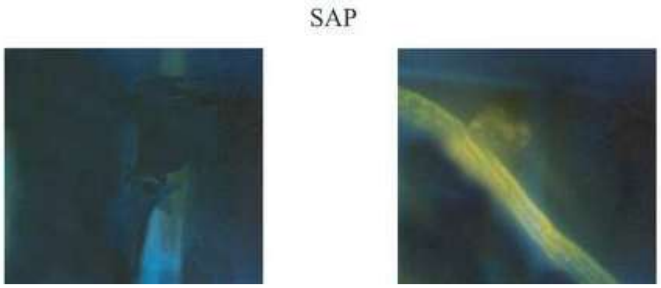

Fig.2. Fluorescence microscopic pictures representative in selective binding to (a) control for SAP (b) SAP in chronic inflammation brain endothelial cell capillaries viewed at a magnification × 40.

3.3. Immunostaining for SAP in the Mice Brain

The SAP accumulation of the brain tissues were further confirmed by immunostaining the brain tissues with antibodies stained against these proteins. Fig. 3b indicates the presence of SAP in the brain tissues. However in the control mice we got the minimal fluorescence in for SAP protein (Fig. 3a).

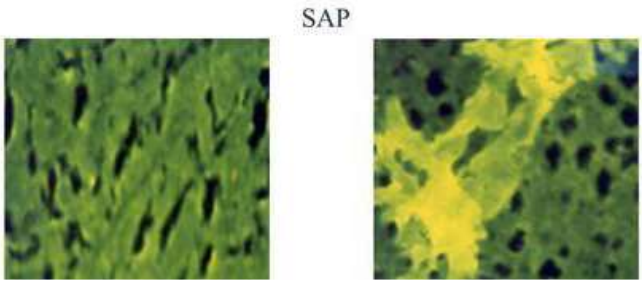

Fig.3: Fluorescence microscopic pictures representative in selective binding to (a) control for SAP (b) SAP in chronic inflammation brain sections viewing field at a magnification × 40.

4. Discussion

The results of the experiment present the following conclusions. First, the SAP proteins are transported across the BBB in the mice with chronic inflammation of systemic amyloid disease. The second conclusion is that the transported SAA along with SAP get localized in the brain. The inflammatory response in pathologically affected regions of amyloid deposition in the brain particularly in AD is well established (Sisodia and Price, 1995). As in the case of peripheral degenerating tissues, damaged neurons and neurite provide stimuli for inflammation. This will lead to up regulation of acute phase reactants and cytokines (Song et al., 2001). Thus, the SAP level increase may be at least partly attributed to the brain tissue expression of these proteins following inflammatory stimulus. The result of the present work suggests that peripheral inflammation significantly contributes to SAP transport across the brain and associated toxic responses.

Endothelial cells of brain capillaries forming the blood brain barrier play an important role in the pathogenesis and therapy of Alzheimer's disease. The modulating effect of SAP on

Abeta in a BBB model was also demonstrated for the first time. SAP, a serum protein belonging to the pentraxin protein family, participates in the regulation of chromatininduced autoimmunity and the immune defense against bacterial infections due to its ability to bind chromatin and bacterial lipopolysaccharides (Erickson et al, 2012). SAP treatment alone did not change brain endothelial paracellular permeability or morphology. In a previous *in vivo* study, when mice were injected with human SAP no deleterious effect on paracellular permeability was noticed. Based on these observations, SAP in physiological concentrations has no effect on brain endothelial TJs (Stanyon and Viles, 2012). SAP avidly binds amyloid fibrils and can be found in all types of amyloid depositions either in the periphery or in the CNS. While SAP is absent from the brain tissue in physiological conditions, it is present in amyloid plaques and depositions around brain vessels in AD and contributes to the stabilization of Abeta fibrils. In our experiments, SAP significantly increased the damaging effect of Abeta on barrier integrity of brain endothelial cells (Delia et al., 2010).

Earlier studies indicate that circulating peptide and protein such as insulin and transthyritin and modified form of catalase gets transported either by adsorption or by facilitated through brain capillary endothelial cell wall (Kusuhara et al., 1997 and Kemper et al., 1999). Under normal condition SAP, which are the normal constituent of blood, which never cross the blood brain barrier (BBB). Coraci et al reported that the class B scavenger receptor (CD 36) on microglial cells binds to fibrillar β-amyloid protein but not its non-fibrillar form (Coraci et al., 2002). Microglial cells get activated when their scavenger receptors bind to fibrillar amyloid. The cellular activation due to SAA fibrils are also implicated to RAGE receptors (Receptor for Advanced Glycation Endproducts) (Sipe et al., 1993 and DeBeer et al., 1993). RAGE is found in most of the cells known to affect by amyloid namely hepatic, endothelial, neuronal, and microglial cells (Yan et al., 2000, Giri et al., 2000 and Yan et al., 1996). Thus, these results suggest that chronic inflammation of systemic amylodosis and central neurons inflammation responses could be interrelated. Serum amyloid P component (SAP) is a normal, constitutive, invariant plasma glycoprotein of the pentraxin family that binds calcium-dependently to amyloid fibrils and is thereby selectively concentrated in all amyloid deposits *in-vivo*. Binding of SAP stabilizes amyloid fibrils and SAP promotes amyloid fibrillogenesis *in-vitro (Kolstoe and wood 2010).*

Our results also indicate that along with SAA fibrils, SAP also crosses the BBB. The presence of SAP in peripheral and neuronal amyloid deposits is well documented (Hawkins et al., 1998)[]. However, the mRNA encoding SAP was not detected in the brain (Kalaria and Grahovae., 1990) indicating that cerebral SAP can be sourced from peripheral region. The present work is consistent with this hypothesis that the SAP, which is found in various inflammatory conditions in the brain, may be due to its crossing of BBB from the plasma. Earlier reports (Upranyie and Medzihradslky) also have shown that the penetrated SAP can be toxic to the cortical neuron.

Conclusion

The significant toxicity of SAP transportation to the brain raising the possibility of alone involved in cerebral neurodegenerative disorders accompanied by amyloid deposition.

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