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Alpha 1 Acid Glycoprotein: Increased Serum and Localized mRNA Expression as a Monitor for Reactions in Leprosy

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Abstract

Alpha 1 Acid Glycoprotein (AGP) has been identified as a potentially useful marker of clinical outcome in disease having an inflammatory component. The objective of this study is to determine the levels of AGP with the possibility of using as a biomarker for Type 1 reactions (T1R) and Type 2 reactions (T2R) in leprosy. Serum levels of AGP are evaluated in a total of 88 leprosy subjects [T1R; n = 32, T2R; n =17 and Non-Reactional (NR); n = 39] using an Enzyme Linked Immunosorbent Assay (ELISA). Relative quantification of mRNA encoding AGP is also done from skin biopsies of same patients using Real-time PCR. The results revealed that the levels of serum AGP are significantly higher in all leprosy cases with T1R and T2R when compared to NR leprosy cases across the study groups ($p < 0.05$). The Cross-sectional analysis also revealed that the levels of mRNA expression of AGP are significantly higher ($p < 0.05$) in leprosy cases with both reactions when compared to non-reactional leprosy Cases ($p < 0.05$). Comparisons are made using Kruskal-Wallis nonparametric test. Significantly higher levels of AGP in serum and in the lesions of patients may indicate its role as a predictive biomarker for type 1 and 2 reactions in leprosy.

Keywords: Alpha 1 Acid Glycoprotein; Biomarker; Leprosy; Type 1 Reaction; Type 2 Reaction

Introduction

Acute inflammation is an essential host responses to tissue injury or infection for the defense and eventual restoration of tissue structure and function. Prolonged inflammation, however, can contribute to the pathogenesis of many diseases leading to loss of the function of tissue or organ which often occurs in leprosy. Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* (*M. leprae*). *M. leprae* mainly resides in macrophages of skin and Schwann cells of peripheral nerves leading to neuropathy and loss of sensation in the extremities of the body and in the area involved in the skin. It is a spectral disease ranging from tuberculoid pole to lepromatous pole with intermediate borderline forms.

Individuals with borderline disease Borderline Tuberculoid (BT), borderline/mid borderline (BB) and Borderline Lepromatous (BL) experience T1R and T2R. T1R is an immunologically mediated episode that is a major cause of nerve function impairment. Nerve function impairment may result in disability and deformity [1]. T2R occurs in Multibacillary (MB) patients (Lepromatous leprosy (LL) and BL) [2]. T2R mainly occurs because of deposition of *M. leprae* antigen-antibody complexes in tissues, which leads to complement activation and neutrophil infiltration resulting in systemic inflammatory response. Both the reactions are mainly responsible for nerve damage that causes permanent disability in leprosy patients.

AGP, also called orosomucoid, are the acute-phase protein that can increase in plasma as much as 5-fold [3] in inflammation during acute and chronic infections. AGP is a 43kDa protein that is highly glycosylated [4]. Due to the presence of sialic acids, AGP is very negatively charged, its pi being only 2.7 [5]. AGP is produced mainly in the liver [6] although some extra-hepatic synthesis of this protein has also been reported [7, 8]. The basal level of AGP in serum is maintained approximately at 20 µmol/L in healthy individuals. During an acute phase condition, the concentration rises 2–5 times, making it one of the predominant proteins in serum. Although AGP is an abundant protein, its real physiological significance is not yet fully understood. Protective effects of AGP against TNF-α induced pathogenesis have been described *In vivo* [9] and gives non-specific resistance against lethal gram negative infection [10]. Keeping in mind both anti-inflammatory as well as the pro-inflammatory property of AGP [11,12] we designed the present study. In the past, there has been one study indicated a high level of AGP in T2R [13] and showed its association with T2R as a biomarker and an indicator of cure during treatment.

It is important that if manifestations of reactions in leprosy are recognized early, then it would be possible for early intervention with protective medicine like steroids and save the nerve and tissue from the damage [14].We, therefore in this study, attempt to examine the role of this protein in the development of both type 1 and 2 reactions in leprosy.

Methods

Study subjects

Eighty eight newly diagnosed untreated leprosy patients with no reaction (NR) (n = 39) and with reactions (n =49; T1R = 32, T2R = 17) (ages between 10 and 60 years) were recruited at TLM Community Hospital, New Delhi, India. All cases were clinically examined by experienced dermatologists and were classified on the histological scale of Ridley-Jopling (RJ) [15] from biopsy specimens.

ELISA for AGP measurement: Serum was separated from 5ml of venous blood followed by addition of protease inhibitor cocktail (Sigma Aldrich Inc.) and stored at −20^{0}C until further processing. Levels of AGP in serum samples were detected in triplicate employing commercial ELISA kit (R&D System Inc. USA) following the manufacturer's instructions.

RNA extraction, cDNA synthesis and quantitative PCR: 5×5 mm incisional skin biopsies taken from the edge of the lesions were collected in RNA later for RNA extraction by using TRI-reagent (Sigma–Aldrich) according to the manufacturer's instructions. The concentration of RNA samples was determined spectrophotometrically at 260/280nm. cDNA was constructed from 1 µg of total RNA from each sample using Protoscript® M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs Inc). Briefly, 1 µg of total RNA was mixed with Random Primer mix and nuclease free water. RNA was then denatured at 70^0 C for 5 min. This is followed by the addition of Reaction Mix containing the buffer, Magnesium ions, dNTPs and Enzyme Mix containing 0.5 units/µl of Reverse Transcriptase and RNase Inhibitor. Temperature cycling conditions included 25° C for 5 min, 45° C for 1 h followed by inactivation of the enzyme at 80°C for 5 min.

Two microliters of cDNA were used per 20 µl reactions. The reaction was carried out using the SYBR Green PCR Master Mix on Rotor-GeneQ (Qiagen Inc. USA) using gene specific primer set designed for this study (Table 1). Each experiment was performed in duplicate.

Statistical analysis

The statistical analysis was performed using Graph-Pad Prism software (Version 6). ANOVA was used to compare means of clinical laboratory parameters. The Kruskal-Wallis nonparametric test was used to test differences between responses among three groups and the post test (Dunn's test) was applied to find out the significant difference between NR and T1R/T2R group. All statistical analyzes were two-sided, and a p value of < 0.05 was considered statistically significant. Graphs are shown as mean ± Standard Error of Mean (SEM) by using Graph pad prism, two-sided table, the n-1 degree of freedom, alpha=0.05, 95% Confidence Interval.

The real-time data were analyzed on Rotor-Gene Q Series Software (Software Version 2.0.2). An association with a p-value < 0.05 was considered as statistically significant.

Results

Serum levels of AGP across the study groups

Analysis of AGP across the study groups reveal that the levels are significantly higher in leprosy cases with T1R and T2R when compared to that of NR group (T1R Vs NR mean± SEM= 464502 ± 26333 vs 231485 ± 22843, T2R Vs NR 450255 ± 41659vs231485 ± 22843, $p < 0.05$ (Figure 1).

Analysis of mRNA expression profiles of GAPDH and AGP in the skin lesions

We analyzed mRNA expression profile by calculating fold difference in expression using Pfaffl method [16] based on the formula mentioned below:

$$Ratio = \frac{\left(E_{target}\right)^{\Delta CT}\ target^{(Control\ -Sample)}}{\left(E_{ref}\right)^{\Delta CT}\ ref^{(Control\ -Sample)}}$$

The percentage efficiency of the primers from the standard graphs was determined to be in the order of 98% for GAPDH (Glyceraldehyde 3 Phosphate Dehydrogenase) (House Keeping Gene) and 97% for AGP.

Individual expression ratio using the above formula was calculated for cases in T1R, T2R and NR leprosy taking the average ratio of NR as the control value for all the cases. Coincident measurement of GAPDH gene has been used for the normalization of target gene expression data.

Cross-sectional analysis of mRNA expression levels of AGP across the study groups revealed that the levels are significantly higher in leprosy cases with T1R and T2R when compared to NR group (T1R Vs NR and T2R Vs NR, mean mRNA Expression Ratios ± SEM = 36.45 ± 19.61 Vs11.02 ± 4.817 and 64.49 ± 17.60 Vs 11.02 ± 4.817, $p < 0.001$). Although no significant difference was found between T1R and T2R (Figure 2).

Discussion

In an attempt to identify a biomarker for detection of the onset of reactions in leprosy we analyzed mRNA expression profile and serum levels of AGP in leprosy patients with type 1 and type 2 reactions along with a control group of patients having no reactions at the time of recruitment. We have identified a statistically significantly high level of AGP in the serum of both types of leprosy reactions. The higher AGP levels as reflected in the serum profile, in active reaction patients than in controls with no reaction could be an early indication of disease progression. AGP is an inflammation related-protein. The several fold increase of AGP concentration in the circulation during an acute phase

Table 1: Primer set for mRNA expression.

Gene	Sequence	Length of Amplicon
GAPDH (Housekeeping gene)	F-5'TTGGTATCGTGGAAGGACTCA-3' R-5'TGTCATCATATTTGGCAGGTTT-3'	270 bp
AGP (in house designed primers)	F-5'CAAGTGACCGCCCATAGTTT3' R-5'GAAAGGCCGATGCGATATAA3'	238 bp

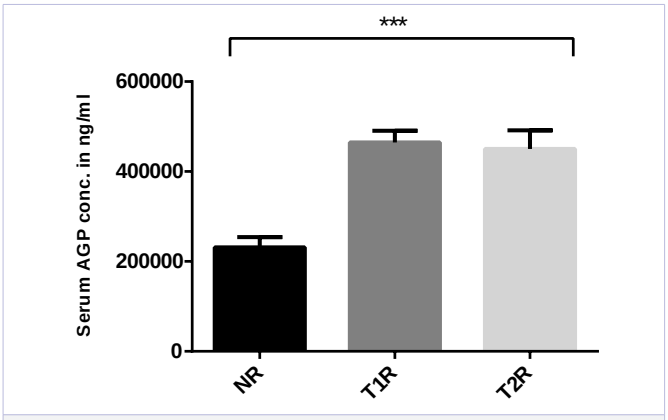

Figure 1: Levels of AGP in serum samples of NR (n =3 9), T1R (n = 32) and T2R (n = 17) of leprosy patients using ELISA. (*** $p < 0.0001$).

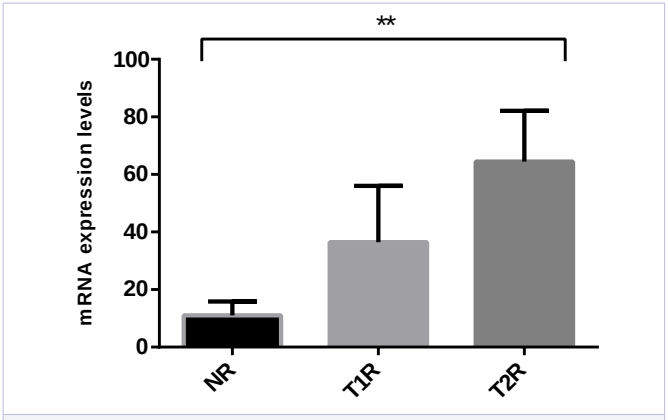

Figure 2: mRNA expression ratios of AGP/GAPDH in lesional skin biopsies of NR (n =39), T1R (n =32) and T2R (n =17) leprosy patients using real time PCR. (** $p < 0.001$)

response could influence the biological functions of the molecule in humans [3]. Although the detailed biological functions of AGP have not been elucidated completely, the major physiological roles of AGP reported so far involve the binding and transport of a range of drugs and it has immunomodulating effects as well [17]. This protein induces monocytes and macrophages to synthesize pro-inflammatory cytokines IL-6, IL-12 and expression of molecules that inhibit the activity of IL-1β and TNF-α by IL-1 receptor antagonist and soluble TNF receptor [18]. This cytokine pattern suggests that AGP might have a dual effect; inducing both a pro-inflammatory environment and an anti-inflammatory environment, depending on the circumstances and the phase of the disease [19]. Leprosy presents a spectrum of immunological groups between the two poles, the tuberculoid pole (TT) with increased cell-mediated immunity, which gradients towards the lepromatous pole (LL) through a series of borderline forms (BT, BB and BL) with simultaneous increase in humoral immune response and increase in the bacillary load. Type1 Reaction (T1R) also called Reversal Reaction (RR), particularly occurs in BT through BL types of leprosy with exacerbations of the pre-existing skin and nerve lesions due to up-gradation of Cell-Mediated Immunity (CMI) more often during and after Multidrug Therapy (MDT) [2]. Dynamics of immunological reactions could reflect changes in the levels of certain circulatory molecules that could act as possible biomarkers for predicting reactions in leprosy (20). A Predictive biomarker for prediction of inflammatory reactions in leprosy has long been desired. In the present study, we have evaluated the diagnostic efficacy of the AGP in T1R and T2R of leprosy that lead to nerve function impairments in the patients. The present study focused on the presence of AGP in circulation and lesions in the reactional state of leprosy. We here demonstrated, using ELISA and Real-Time PCR analysis the presence of AGP, in serum and skin from active lesions of patients with leprosy reactions including skin smear negative BL and BT leprosy patients. Not only systemic but localized levels of AGP measured in terms of relative gene expression revealed that this acute phase protein coding gene is overexpressed in lesional skin mRNA which indicates its active role in regulation of inflammatory episodes at the lesional levels. Although, no significant difference in AGP production was observed between T1R and T2R group. But when taken together, these data suggest that AGP levels correlate with the reactional state in leprosy. However, this study has limitations inherent to its cross-sectional design that cannot determine a causal relationship. It may also be assumed that our sample size is relatively small. More evidence is required to evaluate the association between high levels of AGP, and reactional state of leprosy. Since this study should be considered as preliminary, the consistency of the association might be explored in other clinical studies monitoring the common inflammatory mediators (CRP, IL-6, TNF-α etc.), including AGP, in leprosy patients with T1R, T2R and in patients without any reaction as controls. The present study has several strengths. It is the first study to deal with the association between clinical parameters and T1R, T2R in a leprosy population. We chose untreated leprosy patients with T1R and T2R as a model because it represents the exact role of AGP, in developing the inflammatory condition. To our knowledge, this is the first clinical study demonstrating a systemic link between AGP and inflammation in leprosy using real-time expression for localized along with serum circulatory levels. A prospective cohort study involving measurement of AGP levels, before, during and after the occurrence of T1R, T2R and during MDT treatment may provide predictive information on the effectiveness of AGP as a biomarker to identify patients going to manifest reactions in leprosy.

Conclusion

Acute-phase proteins produced by cytokine activity are useful diagnostic markers that could also be used to monitor treatment response as they can be serially quantified and used as regression markers of the inflammatory response during treatment the quantification of proteins during the course of various acute and chronic inflammatory disorders is useful in diagnosis, therapy, and in some cases, prognosis. The AGP is measurable biomarker that correlates reactions in leprosy and may be indicative factor of conditions favorable to nerve damage, as it shows a significant increase in type 1and 2 reactions both in terms of levels in serum and also in their mRNA expression. The conclusions of our study support the hypothesis that localized, persistent infection may influence systemic levels of inflammatory mediators. However, larger prospective studies are needed to establish its utility as a

biomarker that may be a predictive factor of clinical outcome in the population.

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