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Preliminary data suggest possible association between IL-32 expression level and time of MS attack

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IL-32 and *TNFα* are important cytokines in autoimmune and inflammatory diseases. IL-32 has not been previously studied with respect to MS. Here, we report IL-32 and *TNFα* transcript levels in peripheral blood mononuclear cells of MS patients and control individuals by real time PCR. A significant difference in *IL-32* and *TNFα* mRNA levels of patients as compared to controls was not observed. However, patients with more recent attacks had highly significant increased levels of *IL-32* mRNAs. As the sample size was small, this potentially relevant preliminary observation needs to be corroborated using a larger number of patients with well defined clinical features..© 2011 Progress in Biological Sciences. Vol. 1, No. 2, 44-49.

Introduction

It is generally accepted that cytokines and chemokines are involved in inflammation and autoimmune disease processes, including those pertaining to the central nervous system(Kiyoto et al., 1998, Murphy et al., 2003, Alsaleh et al., 2010).

Although the etiology of multiple sclerosis (MS) is shrouded in unknowns, it is regarded by most as an inflammatory demyelinating autoimmune disease(Hafler and Weiner, 1989). Pro-inflammatory cytokines, including TNFα, TNFβ, IFNγ, IL-4, IL-6, and IL-15 are among immune system related agents believed to have roles in the etiology of MS(Navikas and Link, 1996, KivisÄkk et al., 1998). Relevant observations include presence of pro-inflammatory cytokines in active MS plaques, their increase in the peripheral circulation during active phases of MS, and changes in expression of certain chemokine receptors and chemokines in correlation with clinical course of MS during pregnancy (Beck et al., 1988, Martino et al., 1998, LÃ³pez et al., 2006). TNFα, may be particularly important because it affects permeability at the blood-brain barrier and promotes demyelination(Sharief and Thompson, 1992). IL-32 was originally identified as natural killer cell transcript 4 in 1992, and designated a cytokine in 2005(Kim et al., 2005). It is a pro-inflammatory cytokine that was shown to induce TNFα and to be involved in TNFα related functions(Kim et al., 2005). TNFα has roles in autoimmune and inflammatory diseases such as rheumatoid arthritis and colits, and IL-32 can exacerbate mouse models of these diseases via TNFα(Targan et al., 1997, Shoda et al., 2006). IL-32 is expressed mainly in lymphoid tissues and leukocytes (Targan et al., 1997, Kim et al., 2005, Shoda et al., 2006). It is now known that IL-32 production is induced in response to a wide range of stimuli including IL-18, IL-1β, IFN-γ, and IL-12, and that it can induce several cytokines in addition to TNFα including MIP-2 (macrophage inflammatory protein 2), IL-1, IL-6, and IL-8(Targan et al., 1997, Kim et al., 2005, Goda et al., 2006, Shoda et al., 2006). It has been suggested that

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induction of IL-32 by cytokines and its induction of cytokines creates a positive feedback loop and that IL-32 affects the immune response mainly in this manner(Shoda et al., 2006). There is evidence that IL-32 directly or indirectly affects various functions including phagocytosis and killing of bacteria, prostaglandin E2 related intracellular processes, and apoptosis of T cells(Goda et al., 2006, Conti et al., 2007). This latter function is expected to be relevant in the important process of clearing of activated T cells after the peak of an immune response(Hildeman et al., 2002, Krueger et al., 2003) .

To the best of our knowledge, IL-32 has not yet been studied in MS patients. Here, we measure IL-32 transcript levels in peripheral blood mononuclear cells (PBMCs) of MS patients and control individuals by real time PCR. We also measure the levels of TNFα transcripts in the same individuals. We report that the levels of these transcripts were not significantly different when the cohorts of patients and controls were compared at large. However, there were significant differences in the levels of both IL-32 and TNFα transcripts between subgroups of patients. Specifically, the levels of the transcripts differed between patients with recent attacks and patients without recent attacks. Implications of these observations are discussed.

Material and methods

Patients and control subjects

This research was performed in accordance with the Helsinki Declaration. Initially 13 unrelated Iranian MS patients recruited from MS referral clinics in Esfahan and Tehran in 2010 were studied. The strongly limiting inclusion criterion of not having received any medication or treatment within at least six months prior to recruitment was implemented. Otherwise they were recruited on a consecutive basis. Subsequently, seven additional untreated patients were also recruited. None of the Iranian MS patients in whom serum NO levels were previously reported are included in this study(Roghani et al., 2010). The patients were diagnosed with MS according to the criteria of McDonald et al. (McDonald et al., 2001). The disability status of the patients was rated using Kurtzke's expanded disability status score (EDSS)(Kurtzke, 1983). Fifteen unrelated controls from these cities who self-reported not to be under any medication and to have no indications of disease were also recruited from the same cities.

Real time PCR

PBMCs were separated from 4cc blood of participants by Ficoll-Isopaque density centrifugation (Gibco BRL, Life Technologies Ltd, UK). RNA was isolated from the cells using RNX+ according to the manufacturer's instructions (Cinnagen Tehran, Iran). RNA yield was determined spectrophotometrically and RNA quality was assessed after electrophoresis by using density ratio of 28S to 18S ribosomal RNA bands. Complementary DNA (cDNA) was synthesized with the Bioneer cDNA synthesis kit (Alameda, CA, USA). Candidate control genes for assessment of levels of *IL-32* and *TNFα* mRNA levels were *ACTB*, *GADPH*, and *B2M*. Real time PCR on the candidate control genes was done using cDNA of 5 patients and 6 control individuals on an ABI7500 machine (Foster City, CA, USA) using the QuantiFast SYBR Green PCR Kit (QIAGEN; Germantown, MD, USA). Results of the real time experiments were submitted to geNorm program (http://medgen.ugent.be / ~ jvdesomp/genorm/) for identification of internal control gene with least variable transcript level among the samples. Subsequently, real time PCRs for *IL-32* and *TNFα* were performed on the cDNAs of the 13 patients and 15 control individuals. Real time PCR was done only for *IL-32* on the cDNA of the 7 patients later recruited. All gene specific primers for real time experiments were purchased from QIAGEN (*ACTB*: QT01680476; *GADPH*: QT01192646; *B2M*: QT00088935; *IL-32*: QT00199766; *TNFα*: QT01079561). Expression levels of *IL-32* and *TNFα* between patients and controls were compared by comparing their ΔCT values, and comparison of expression of the genes between patient subgroups was done by comparing $2^{-\Delta\Delta CT}$ values(Livak and Schmittgen, 2001).

Statistical analysis

Statistical analysis was done using the student's t-test and analysis of variance (ANOVA).

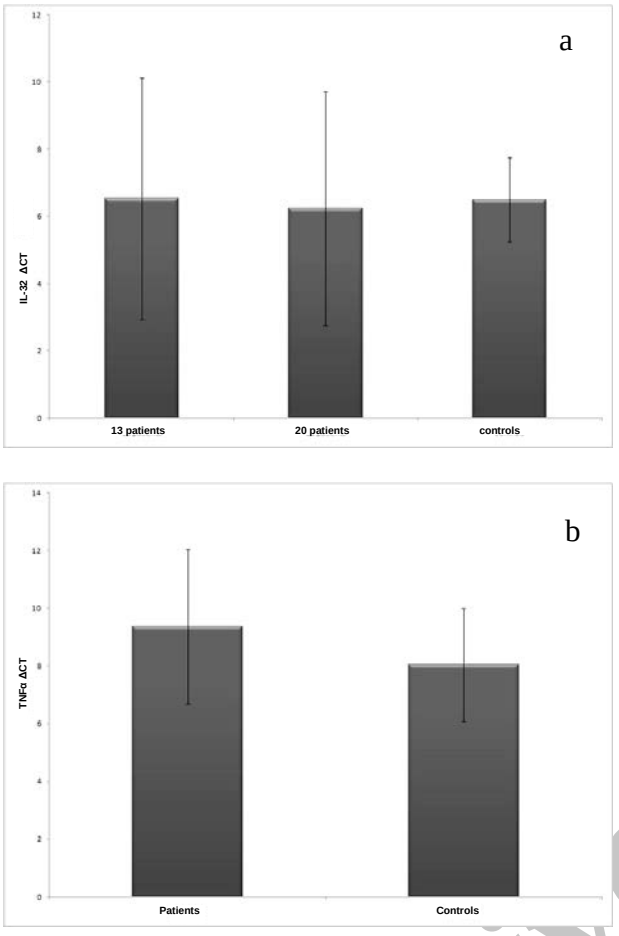

Fig. 1. Comparison of mean *IL-32* (A) and *TNFα* (B) mRNA levels between MS patients and controls.

The SPSS 17.0 and Minitab 15 statistical program software were used.

Results

The clinical features of the patients are presented in Table 1. The fact that almost all our patients were women is a reflection of the notably skewed female to male ratio among Iranian MS patients(Roghani et al., 2010). Course of disease in the vast majority of our patients was relapsing-remitting, consistent with previous reports from Iran (Roghani et al., 2010).

GeNorm identified *ACTB* as the gene with the best stability value (M=0.076). *IL-32* and *TNFα* mRNA levels were not significantly different between the cohorts consisting of 13 MS patients and 15 control individuals (p=0.70 and 0.20, respectively; Fig. 1A,B.). Absence of significant difference in *IL-32* levels was still apparent after inclusion of data pertaining to seven patients recruited in 2010 (p=0.61; Fig. 1A.). The raw data on *IL-32* and *TNFα* mRNA levels showed clustering of the values for each gene among control individuals and dispersion among the patients. This is reflected in the relatively large standard deviations associated with the average values for patients (*IL-32* ΔCT: 6.24+/-3.48; *TNFα* ΔCT: 9.38+/- 2.67) as compared to control individuals (*IL-32* ΔCT: 6.50+/-1.25; *TNFα* ΔCT: 8.04+/- 1.97). Dispersal of TNFα and other cytokine levels at the protein level is also evident in the data presented by other authors(Kiyoto et al., 1998).

We thought this may suggest heterogeneity among the MS patients and that the levels of some of cytokines may differ among patient subgroups.

We observed no correlation between levels of *IL-32* and *TNFα* mRNAs and EDSS scores of the patients (*IL-32*: Pearson correlation =0.142, p=0.50; *TNFα*: Pearson correlation =-0.426, p=0.15). There was also no significant correlation between the Progression Index (EDSS changes per year) of the patients and *IL-32* and *TNFα* mRNA levels (*IL32*: Pearson correlation=0.035, p=0.890; *TNF-α*: Pearson correlation+-0.318, p=0.340). Subsequently, we compared the levels of the mRNAs between relapse-remitting (RR) patients who had and who had not experienced a relapse within two months before or after time of sample collection. Among the 15 and 10 RR patients in whom, respectively, *IL-32* and *TNFα* were measured, information on time of most recent relapse on 12 and 10 were available. Based on 2-ΔΔCT values, *IL-32* mRNA levels were significantly higher (p=0.008) among the six patients who had had a relapse within this time period as compared to the six who had not had a relapse (Fig. 2A.). *TNFα* mRNA levels were also significantly higher (p=0.013) among the four patients who had had a relapse within this time period as compared to the six who had not had a relapse (Fig. 2B.). Notably, the levels of *IL-32* and *TNFα* mRNAs in primary progressive (PP) and secondary progressive (SP) patients were similar

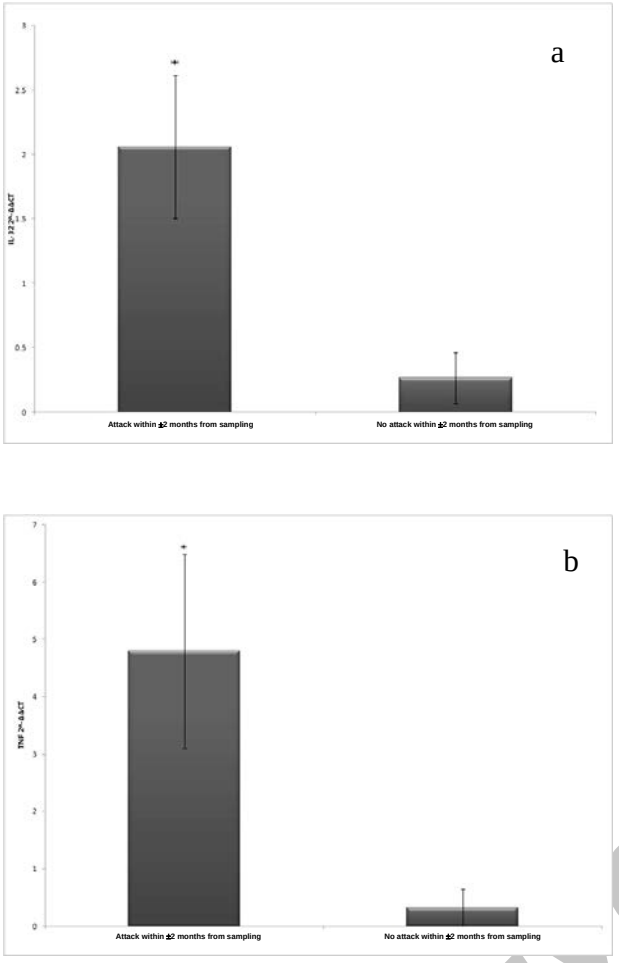

Fig. 2. Comparison of *IL-32* (A) and *TNFα* (B) mRNA levels between relapsing remitting MS patients with and without attack in period +/- two months of blood withdrawal.

to RR patients without recent attacks, and significantly lower than RR patients with recent attacks (not shown); clearly PP and SP patients have not experienced a recent attack. The correlations we have were always in the same direction for *IL-32* and *TNFα* mRNAs. Consistent with this, we observed a close correlation between *IL-32* and *TNFα* mRNA levels among both our patient (Pearson correlation =0.920, p=0.00) and control (Pearson correlation =0.985, p=0.00) cohorts. Finally, there was no significant correlation between age at onset (*IL-32*: Pearson correlation =0.059, p=0.85; *TNFα*: Pearson correlation =-0.176, p=0.57) or disease

Table 1. Clinical features of MS patients

	TNF-α	IL-32
No. of patients*	13	20
No. females	12	19
Average age at onset	29.0±6.3 yrs.	28.9±7.1 yrs.
Average age at examination	35.5±8.8 yrs.	35.3±8.9 yrs.
Duration	6.4±7.1 yrs.	6.8±6.8 yrs.
Average EDSS score	2.42±1.8	2.7±2.0
Clinical course**		
RR	10	15
SP	1	1
PP	2	4

*the 20 patients include 13 originally recruited and 7 later recruited.

**RR, relapsing-remitting; SP, secondary progressive; PP, primary progressive.

duration (*IL-32*: Pearson correlation = -0.192, p=0.53;*TNFα*: Pearson correlation =-0.351, p=0.24) and *IL-32* and *TNFα* mRNA levels.

Discussion

IL-32 levels at the mRNA or protein levels have been shown to be lower in controls as compared to patients affected with various autoimmune and inflammatory diseases including rheumatoid arthritis, myeloblastic leukemia, HIV,and Crohn's disease(Dinarello and Kim, 2006, Marcondes et al., 2008, Rasool et al., 2008, Alsaleh et al., 2010). We did not observe a significant difference in *IL-32* mRNA levels between MS patients and controls.

We expect medical treatments were not relevant to these observations as none of our patients had received recent treatment. In most previous studies on other diseases, IL-32 was measured in the target tissue of the disease e.g. the synovial tissue in rheumatoid arthritis and in intestinal epithelial cells in Crohn's disease(Dinarello and Kim, 2006, Shioya et al., 2007, Alsaleh et al., 2010). The fact that we did not have access to MS target tissue and used PBMCs as surrogate may partly explain why we were unable to detect a difference. Perhaps more important is the consideration that cytokines including IL-32 may exhibit an expression pattern that is not uniform at all stages of disease activity. We observed a more non-uniform distribution of *IL-32* mRNA levels among our patients as compared

to controls. Grouping our patients based on some MS relevant parameters showed that they did not correlate with *IL-32* levels. Notably, there was no significant correlation with EDSS scores or with Progression Index. However, grouping based on proximity in time to attack did show statistically significant differences in *IL-32* expression. Patients who had recently or were about to experience attacks had higher levels of *IL-32* mRNAs in their PBMCs. The statistical significance of the correlation (p=0.008) was notable.

TNFα is a multifunctional cytokine with important roles in the immune system (Cope, 1998). It is considered a driving factor in the demyelination process associated with MS(Lock et al., 1999). *IL-32* and *TNFα* levels have been shown to be concordant in several studies and it has been suggested that [i]interactions between *TNFα* and *IL-32* are important in the functions of both cytokines in various disease conditions (Kim et al., 2005). It was because of these considerations that we measured *TNFα* mRNA levels while also measuring *IL-32* mRNA levels.

Consistent with observations in other autoimmune and inflammatory diseases, we observed a strong correlation between the expressions of these two cytokines. During the couple of months before and after attack, there were increases in the levels of both. These preliminary results suggest that monitoring the levels of these cytokines in easily accessible PBMCs might be informative for prediction of oncoming attacks and may suggest targets for effective anti-infalmmatory treatments for prevention or control of attack. Similarly, they may be tools for monitoring effectiveness of treatment protocols. Although our results are interesting, our sample size was small and the data need to be corroborated using a larger number of patients with well defined clinical features. Although our results are interesting, our sample size was small due to patient recruitment criterion of not having received recent medication. The preliminary findings need to be corroborated using a larger number of patients with well defined clinical features.

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