Research article – Histology and cell biology

Serum relaxin levels in subjects with multiple sclerosis

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Abstract

Multiple Sclerosis is an inflammatory, auto-immune, neurodegenerative disease of the central nervous system. The disease has a prevalence of approx 1:700 with at least 2.5 million cases worldwide. It is the leading, non-trauma cause of physical disability among young and middle-aged adults. Recently developed therapies do reduce disease activity but only modestly, with all of the available agents producing significant side effects that reduce compliance, and/or serious risk for adverse events. Relaxin has been long-recognized to play a critical role in pregnancy. Recent investigations have revealed that relaxin may be an important regulator of inflammation and immune processes. This is due to the ability of relaxin to promote the production of glucocorticoid receptors, increase serum levels of the adrenocorticotropic hormone and inhibiting cell-mediated pro-inflammatory activity by stimulation of the peroxisome proliferator-activated receptor gamma. This study found serum relaxin levels to be elevated in subjects with multiple sclerosis. Production of relaxin is down regulated by a negative feedback loop through its own receptor binding. Decreased receptor binding may contribute to the higher level of relaxin seen in these patients and may lead to dysregulation of the inflammatory and immune pathways.

Key words

Inflammation, auto-immunity, pregnancy, peroxisome proliferator-activated receptor gamma, glucocorticoids

Key to abbreviations

ACTH   Adrenocorticotropic Hormone
CNS   Central Nervous System
DNA   Deoxyribonucleic Acid
EAE   Experimental Allergic Encephalomyelitis
GCR   Glucocorticoid Receptor
IFNγ Interferon Gamma
MS   Multiple Sclerosis
IL   interleukin
PPARγ Peroxisome Proliferator-activated Receptor Gamma
RLX   Relaxin
RXFP-1 Relaxin Family Petide Receptor-1
TNFα Tumor Necrosis Factor Alpha

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Introduction

Multiple sclerosis (MS) is an inflammatory, auto-immune disease of the central nervous system (CNS). It has a world-wide in distribution, with more than 2.5 million cases. It is the cause of eventual severe disability in 75-85% of subjects developing the disease (Weinshenker, 1998; Kantarci and Weinshenker, 2005). The recognition that MS is a largely an inflammatory, auto-reactive lymphocyte-driven disease has led to the development of therapeutic strategies that have resulted in a decrease in the risk of clinical relapses, reduction in the progression of lesion burden on magnetic resonance imaging scans, and in some cases reduced disability progression over the short term, generally for 2 to 3 years (Bornstein et al., 1987; Jacobs et al., 1996; PRISMS Study Group, 1999; Durell et al., 2002). It is not known whether there is significant therapy-induced long term reduction in risk of disability progression, although there are some recent data supporting the proposal that relapse reduction, particularly early in the disease course, may be associated with less disability progression (Bermel et al., 2010).

It is well-known that multiple sclerosis clinical disease activity abates during pregnancy (Lorenzi and Ford, 2002). Although the mechanism(s) of these changes are not fully understood they are thought to be due to the rise in estrogen levels during pregnancy (Gold and Voskuhl, 2009). Early clinical trials using the estrogen β-estradiol in MS have shown encouraging results (Sicotte et al., 2002) and several phase II studies are currently underway (Gold and Voskuhl, 2009).

One possible mechanism of action for β-estradiol is through its ability to up-regulate the expression of relaxin 2 (RLX2), a peptide member of the insulin superfamily (Lao Guico and Sherwood, 1985). Other members include RLX1and RLX3. RLX1 and RLX2 have been shown to bind the same receptors, but are differentially expressed (Hansell, 1991). Both RLX1 and RLX2 are up-regulated by glucocorticoids, but only RLX2 is up-regulated by sex hormones, suggesting different biological roles for RLX1 and RLX2 (Garibay-Tupas et al., 2004). In contrast, RLX3 is expressed primarily in the brain where is acts as a neurotransmitter and is involved in feeding and stress responses (Bathgate et al., 2013).

RLX2 is detected transiently in the plasma of both men and women (Moriatis Wolf et al., 2013) and has been studied extensively in pregnancy, cardiovascular disease and renal disease (Bathgate et al., 2013). From these studies we know that RLX2 is a pleiotropic protein which exhibit enhanced binding in the presence of β-estradiol (Wilson et al., 2005; Santora et al., 2007; Figueiredo et al., 2009). The receptors for RLX2 (and RLX1) are RLX family peptide receptors (RXFP-1 and RXFP-2), the glucocorticoid receptor (GCR) and estrogen receptor beta (ERB) (Dschietzig, et al. 2004; Sherwood, 2004). These regulate pathways which are currently being researched in MS.

Binding to RXFP-1 increases PPARγ transcription activity via RXFP-1 (Hsu SY et al., 2002, Bathgate et al., 2006) without increasing PPARγ mRNA or PPARγ protein levels (Singh and Bennett, 2010). This is a pathway of great interest in MS because other PPAR agonist, such as pioglitazone and rosiglitazone also have antiglycemic and anti-inflammatory properties (Bright et al., 2008), inhibit mitogen-stimulated T-cell proliferation as well as tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) production (Schmidt et al., 2004). In experimental allergic encephalomyelitis (EAE), an animal model of MS, pioglitazone reduce lymphocyte proliferation and

infiltration into the CNS (Niino et al., 2001), blocks interleukin (IL)-12 induced differentiation of pro-inflammatory CD4+ helper T cells into type 1 helper cells (Shevach et al., 1999), reduces gene expression for IL-1β and IL-6, reduces production of TNFα and IFNγ and reduces clinical disease expression (Feinstein et al., 2002; Natarajan and Bright, 2002; Klotz et al., 2005). In addition, PPARγ gene knockout mice are more susceptible to developing EAE (Natarajan et al., 2003) and in limited studies in MS patients PPARγ expression appeared reduced in peripheral immune cells and these cells have reduced responsiveness to PPARγ agonists (Pershadsingh et al., 2004).

Binding to GCR elicits a potent anti-inflammatory and immuno-regulating response (Newton, 2000). These have been well studied in MS and GCR agonists are the primary approach to treating acute attacks of the disease (Citterio et al., 2000).

In vitro studies of ERB agonists have shown that they have neuroprotective effects (Wisdom et al., 2013) and the ability to promote remyelination (Crawford et al., 2010). Agonists of the ERB have been primarily studied in MS through the actions of sex hormones. These studies have shown that binding of estrogen to ERB is one mechanism through which estrogen treatment was neuroprotective and promoted recovery from exacerbations (Gold and Voskuhl, 2009).

The interest in a possible role for RLX in MS was further supported by chromosomal localization of both RLX1 and RLX2 at 9p24.1 (Garibay-Tupas et al., 1999), an area identified in linkage studies as a susceptibility locus for MS (Patsopoulos and de Bakker, 2011). The location of the RLX genes at 9p24.1 together with their diverse actions supported the investigation of RLX in subjects with MS.

Materials and methods

Sample Collection

Serum and plasma samples were obtained from controls (n=14, 8 females and 4 males, aged 36-62 years) and MS patients (n=16, 10 females and 6 males, aged 34-64 years) by venipuncture, randomized and assayed blind. The study and consent forms were approved by the internal review board.

Relaxin Levels

Relaxin levels (pg/ml) were measured in serum by ELISA (Immundiagnostik AG, Germany) according to the manufacturer's instructions.

DNA Sequencing

Isolation of genomic DNA samples was carried out using the QIAamp DNA Blood Mini Kits (Qiagen) according to the manufacturers protocol. Briefly, 200 μl of buffy coat was added to a solution of Proteinase , RNase A and lysis buffer. The mixture was incubated at 56°C for 10 minutes. Next, 200 μl of 100% ethanol were added to the sample and mixed pulse-vortexing for 15 seconds. The solution was then applied to a QIAamp spin column (Qiagen) placed in a 2 ml collection tube and centrifuged at 6000 x g for 1 minute. The spin column was then placed in a clean 2 ml

collection tube and re-centrifuged at full speed for 15 seconds. Wash buffer 1 (500 μl) was added to the column and centrifuged at 6000 x g for 1 minute. The spin column was placed in a clean 2 ml collection tube and 500 μl of wash buffer 2 was added. The column was then centrifuged at full speed for 3 minutes. The spin column was then placed in a new 2 ml collection tube and centrifuged at full speed for 1 minute. Finally, the spin column was placed in a clean 1.5 ml microcentrifuge tube and 200 μl of distilled water were added to the column. After incubating at room temperature for 5 minutes, the DNA was eluted by centrifugation at 6000 x g for 1 minute. The eluate was collected and stored at -80^{0}C prior to sequencing. The target genes (RLX1 & RLX2) were then sequenced using published primer pairs (Fu and Evans,1992).

Protein Modeling

The binding affinity of RLX has been shown to be dependent on conformational and electrostatic forces (Bathgate et al., 2013). To determine if changes in the DNA sequence of RLX could affect the receptor binding region, protein modeling was done. These models were based on the known structure of human RLX using Swissprot (Arnold, 2006; Bordoli, 2009).

Results

Serum RLX levels

Relaxin was not detected in the control group. However, there were two distinct cohorts in the MS group. RLX was detected in cohort 1 (56% of subjects) but not detected in cohort 2 (44% of subjects, see Tab. 1). The elevated RLX levels did not correlate with differences in age, sex or duration of disease. But there was a moderate correlation between RLX levels and weeks since last exacerbation. Correlation coefficient was calculated by the Pearson method (r = $\sum((X - M_y)(Y - M_x)) / \sqrt{((SS_x)(SS_y)}$ R = -0.6825, P-Value = 0.043008. (see Fig. 1).

DNA Sequencing

No mutations were detected in the sequence for RLX2. However, there were a number of sequence changes in the gene for RLX1:

Table 1 - Patient characteristics and relaxin serum concentration.

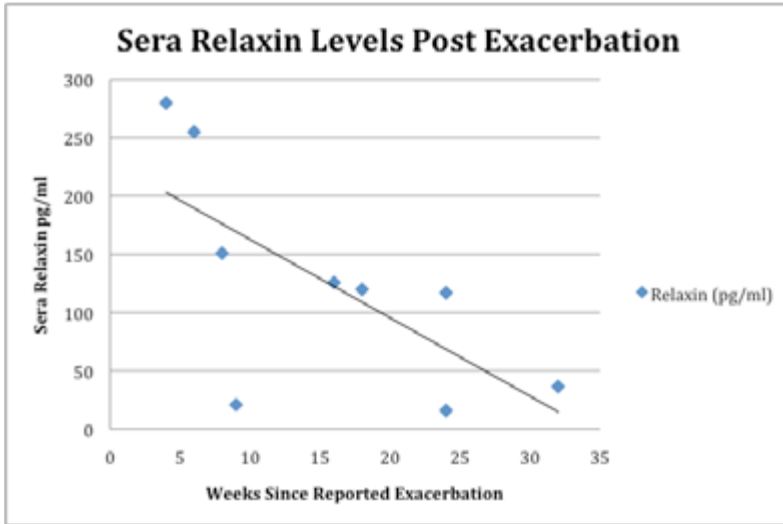| Group	Disease Duration (years) Mean (range)	Sex	Age (years) Mean (range)	Serum relaxin (pg/ml)
MS Cohort 1	13.1 (7-19)	5 F, 4 M	45 (39-56)	124.54 (15.79-279.88)
MS Cohort 2	12.0 (1-32)	5 F, 2 M	39 (34-64)	0
Control		8 F, 4 M	50 (36-62)	0

Figure 1 – Subjects in cohort 1 had measurable levels of relaxin in the sera and there was a moderate correlation with the amount of time since last exacerbation. Correlation co-efficient was calculated by the Pearson method; R = -0.6825, P-Value = 0.043008.
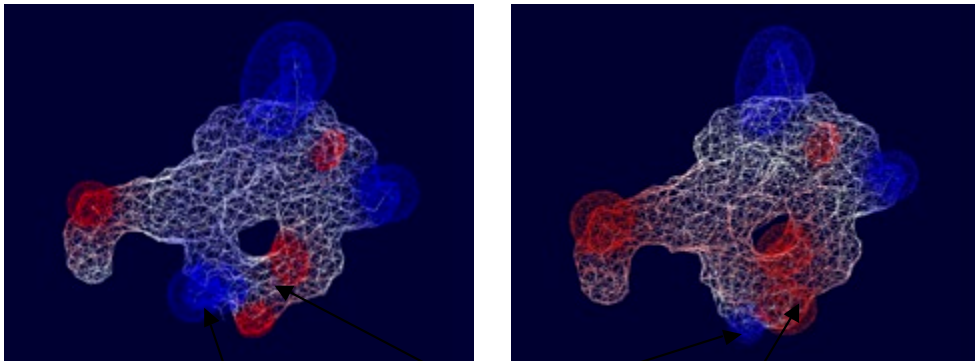

Figure 2 – Conformational and electrostatic changes in the B chain binding cassette of relaxin 1 from subjects with MS compared to controls. Blue indicates positive charge, red indicates negative charge. Changes in the electrostatic forces of relaxin 1 could potentially affect the ability of relaxin to bind to its receptors.

RLX1-WT = L E F C L L L N Q F S R A V A A K W K D D V I K L C G R E L V
RLX1-MS = L **G V** C L L L N Q F S R A V A **D S** W **M E E** V I K L C G R E L V

Protein modeling

Protein modeling suggests that the changes in the amino acid sequence could affect RLX1 conformation and electrostatic forces (see Fig. 2).

Discussion

Relaxin levels and activity were measured in sera from non-pregnant subjects with MS and controls. These results showed that serum RLX levels were detectable in 56% of subjects with MS and not detected in any of the control samples. The highest serum RLX levels were detected in those who had experienced recent inflammatory events, although this was a weak correlation. Some of the subjects expressing RLX had levels of RLX in the sera usually only observed in pregnancy (>250 pg/ml). The circulating RLX that we detected could be either RLX1 or RLX2, or a combination of both. The reason for its presence is unclear but could possibly be due to a number of factors.

Chronic cerebral inflammation is a hallmark of MS and is transiently present in both relapsing remitting and progressive forms of the disease (Weiner, 2009). Inflammation increases the expression of glucocorticoids (Black, 2002) which in turn can up-regulate the expression of RLX1 and RLX2 (Garibay-Tupas, 2004). The underlying presence of chronic inflammation in subjects with MS could explain the detection of relatively high levels of circulating RLX in these subjects. Another possible source of RLX in the circulation might be from RLX expression in the brain. The RLXs produced in the CNS might migrate through the blood brain barrier which is disrupted in MS (Minagar and Alexander, 2003).

Another possible factor contributing to elevated levels of circulating RLX is the disruption of auto-regulation through binding to GCR. Auto-regulation has been described in this way for RLX2 (Dschietzig et al., 2009). Since RLX1 and RLX2 are both regulated through the GCR they may share this common auto-regulation. The mutations described in the gene encoding RLX1 in MS might disrupt GCR binding and any auto-regulation of RLX1 production through this mechanism.

The presence of circulating RLX outside of pregnancy suggests a role for RLX in other than reproductive biology. Perumal and Dhanasekaran (2014) recently suggested a role for RLX in systemic lupus erythematosus. Lupus is an inflammatory, auto-immune disease which has been genetically linked to MS (Mandel et al., 2004). To clarify the role of RLX, if any, in MS further research needs to be undertaken to identify the circulating RLX and its source. It is possible that sera RLX is a biomarker of inflammation, due to leakage across the blood brain barrier or its presence might be due to disrupted auto-regulation. In any case, it presents a new pathway of research in MS. And further studies of a possible role of RLX in MS may provide targets for the development of novel therapeutics.

Conflict of Interest

The authors have no conflict of interest to declare.

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