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Relaxin levels in matched sera and CSF from subjects with multiple sclerosis

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Abstract

A spontaneous remission of symptoms in women with multiple sclerosis is often associated with pregnancy. Sex hormones such are thought to play a crucial role in this. Both testosterone and estrogen have been studied extensively in the disease, but not relaxin. In an initial study of relaxin in multiple sclerosis elevated levels were detected in the sera of some, but not all subjects. Unfortunately, it was not possible to determine if this was relaxin 1, 2 or 3 due to the non-specificity of the assay. In this study we measured the levels of all three relaxins in matched sera and cerebral spinal fluid samples from subjects with multiple sclerosis. No relaxin 2 was detected in any of the samples. However, both relaxin 1 and 3 were detected in some of the samples. The presence of relaxin 3 was not remarkable. In contrast, the presence of relaxin 1 was notable. The presence of relaxin 1 in subjects with multiple sclerosis suggests that it has a biological function and is not a pseudogene has been previously suggested.

Key words

Relaxin, multiple sclerosis, sera, cerebral spinal fluid.

Introduction

Multiple sclerosis (MS) is an inflammatory, autoimmune disease of the central nervous system (CNS). The disease course of MS can be either a relapsing, remitting or progressive form (Goldberrg, 2012). In women with the relapsing, remitting form of MS, the disease symptoms often go into spontaneous remission during pregnancy (Lorenzi and Ford, 2002). This has engendered research into the role of sex hormones in MS, most notably estrogen (Gold and Vosckuhl, 2009). Estrogens have also been shown to work synergistically with another sex hormone, relaxin (RLN, Huang and Anderson, 1997) and can regulate the mRNA expression of the relaxin receptor 1 (RXFP1, Maseelall, 2009). Since RLN is expression is increased during pregnancy, could its synergistic actions with estrogen play a role in the remission of symptoms?

There had been no studies on RLN in subjects with MS published before. Thus a baseline study was undertaken to determine the levels of circulating relaxin in non-pregnant subjects with MS. In this study we reported on measurable levels of the pregnancy hormone RLN in the sera of some subjects with MS (Garvin and Burns, 2016). However, the ELISA used to detect RLN in these samples was non-specific and detected both RLN 1 and 2. Because of this it was unclear if the RLN detected in these samples was RLN 1 or 2.

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In the present study we used ELISA kits specific for RLN 1, 2 and 3 to determine the levels of each member of the RLN family in matched sera and cerebral spinal fluid (CSF) samples from subjects with MS.

Methods

Samples

Sera and CSF samples were obtained from the Rocky Mountain Multiple Sclerosis (RMMS) Tissue Bank, Denver, Colorado (see Table 1). Prior to obtaining the samples, the project was reviewed and approved by the RMMS ethics review board. The samples analyzed in this study were obtained at postmortem, with family consent from subjects with MS (n=12) and controls (n=4). Sera and CSF samples were then held at -80^{0}C until analysis.

Table 1. Characteristics of sample donors.

Sample	Diagnosis	Sex	Age	PM (hrs)
1	Control	F	55	N/A
2	Control	M	67	N/A
3	Control	F	43	3
4	Control	F	61	5.5
5	MS	F	59	4.5
6	MS	F	41	N/A
7	MS	F	64	5.5
8	MS	F	69	7
9	MS	M	55	N/A
10	MS	F	82	4
11	MS	F	77	N/A
12	MS	M	45	2
13	MS	F	67	4.5
14	MS	F	35	3
15	MS	F	84	2.5
16	MS	F	64	4

Table of sera and CSF (cerebro-spinal fluid) donors. The sample consisted of 4 controls (3 females, 1 male) average age of 56.5 years. The MS (multiple sclerosis) subset consisted of 12 subjects (10 female, 2 male) with and average age of 61.8. Samples were collected post mortem (PM) and stored at -80^{0}C until assayed.

Enzyme Linked Immuno-absorbent Assays (ELISA)

DuoSet ELISA kits for the detection of human relaxin 1 & 3 were ordered from R&D Systems Inc., 614 McKinley Place NE, Minneapolis, MN. In addition to the materials provided the following solutions were required:

- Phosphate buffered saline (PBS) – 137mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 in distilled, deionized water. Solution was adjusted to pH 7.2-7.4 and filtered through a 2mm filter.
- Wash Buffer – 0.05% Tween$^{\text{Ò}}$ 20 in PBS, pH 7.2-7.4.
- Reagent Dilluent – 1% Bovine serum albumin in PBS, pH 7.2-7.4 filtered through a 2mm filter.
- Substrate Solution – 1:1 Mixture of hydrogen peroxide and tetramethylbevzidine.
- Stop solution – 2 N H_2SO_4.

The DuoSet ELISA kits for human RLN 1 & 3 are sandwich ELISA's and followed the same protocol with the exception of the capture and detection antibodies employed.

For RLN 1 the capture antibody used was rat anti-human RLN 1, 4.0 mg/ml in PBS, pH 7.2-7.4 and the detection antibody was biotinylated goat anti-human RLN1, 400 ng/ml in reagent diluent.

For RLN 3 the capture antibody used was goat anti-human RLN 3, 1.2 mg/ml in PBS, pH 7.2-7.4 and the detection antibody was biotinylated goat anti-human RLN 3, 400 ng/ml in reagent diluent.

The capture antibodies were supplied as lyophilized powder and were reconstituted in PBS then diluted with PBS to the working concentration just prior to use.

The ELISA's were performed according to the manufacturers instructions.

Plate Preparation

1. After reconstitution 100 ml of the appropriate capture antibody was added to each well in a 96 well microtitre plate.
2. The plates were sealed and incubated overnight (ON) at room temperature (RT). The antibody solutions were aspirated off and the plates washed three times with 400 ml of wash buffer using an autowasher. Any remaining fluid in the plates was removed by inverting and blotting the plate against a paper towel.
3. The plates were blocked by adding 300 ml of reagent diluent to each well. The plates were incubated at RT for 1 hour.
4. Plates were aspirated and washed on the autowasher and blotted dry as described.

Assay Procedure

1. A plate layout was followed to add 100 ml of the appropriate samples or standards to each well. The plate was then covered with a new adhesive strip and incubated at RT for 2 hours.
2. The plates were aspirated, washed on the autowasher and blotted dry as described.
3. Then 100 ml of the appropriate detection antibody was added to each well. The plates were sealed with a new adhesive strip and incubated at RT for 2 hours.
4. Plates were aspirated and washed on the autowasher and blotted dry as described.
5. The streptavidin conjugated to horse radish peroxidase (STD-HRP) supplied by the manufacturer was diluted 1:100 in diluent buffer and 100 ml added to each well. The plate was then covered with a new adhesive strip and placed in a dark chamber for 20 minutes at RT.
6. Plates were aspirated and washed on the autowasher and blotted dry as described.
7. After washing 100 ml of substrate solution was added to each well. The plates were covered with a new adhesive strip and incubated in a dark chamber for 20 minutes at RT.
8. Finally, 50 ml of stop solution was added to each well. The plate was lightly tapped on the side to ensure even mixing of the solutions.
9. The plates were immediately read at a wavelength of 450 nm in an auto-reader (Hewlett Packard, Palo alto, CA) using a wavelength correction reading at 540 nm.

RLN 2 ELISA

The RLN 2 Quantikine ELISA kit was obtained from R&D Systems Inc., 614 McKinley Place NE, Minneapolis, MN. The Quantikine ELISA was supplied with all reagents and pre-coated plates needed for the assay.

Assay procedure

1. To each well 100 ml of diluent RD1-19 was added. 50ml of the appropriate standard, control or sample was then added to each well. The plate was covered with an adhesive strip and incubated for 2 hours at RT.
2. Plates were aspirated and washed with the wash solution provided on the autowasher, then blotted dry as described above.
3. To each well 200 ml of anti-RLN 2 antibody conjugated to HRP was added. The plate was covered with a new adhesive strip and incubated for 2 hours at RT.
4. Plates were aspirated and washed on the autowasher, then blotted dry as described above.
5. To each well 200 ml of substrate solution was added. The plate was covered with a new adhesive strip and incubated in a dark chamber for 30 minutes at RT.
6. After the final incubation 50 ml of stop solution was added to each well.
7. The plates were immediately read at a wavelength of 450 nm in an auto-reader (Hewlett Packard, Palo Alto, CA) using a wavelength correction reading at 540 nm.

Statistical Analysis

ELISA analysis was done using the internet based software provided by ELISAkit.com, Melbourne, Australia, http://elisaanalysis.com/. Concentrations were calculated from OD's using 4-Parameter Logistic Regression. All significant results had a value of $p<0.05$.

Results

Measurable levels of RLN were detected in 6 of the MS subjects. No measurable levels of RLN were detected in any of the controls.

RLN 1 was detected in the sera and CSF of some subjects with MS (see Table 2). There was measurable RLN 1 in the sera of one subject (179, $P<0.05$) and in the CSF of two others (147 ng/ml, $P<0.05$ and 212 ng/ml, $P<0.05$).

RLN 2 was not detected in the sera or CSF of any sample.

RLN 3 was detected in sera and CSF from subjects with MS (see table 2). There was measurable RLN 3 in the sera of one subject with MS (100 ng/ml, $P<0.05$) and in the CSF of two further subjects with MS (1.318 ng/ml,$P<0.05$ and 12.587 ng/ml, $P<0.05$).

Discussion

The presence of RLN3 in both the sera and CSF of subjects with MS could be due to production in the CNS and by adipocytes (Yamamoto et al, 2014). Although most of the post mortem samples used in this study were collected between 2-5.5 hours after death. It is possible that the absence of measurable relaxin levels in some subjects could be due to degradation.

Table 2. Relaxin Levels in Sera and CSF.

Sample	Diagnosis	RLN 1 (ng/ml)				RLN 3 (ng/ml)			
		Sera	P	CSF	P	Sera	P	CSF	P
1	Control	-		-		-		-	
2	Control	-		-		-		-	
3	Control	-		-		-		-	
4	Control	-		-		-		-	
5	MS	-		-		-		-	
6	MS	-		-		-		1.318	P<0.05
7	MS	-		-		-		12.587	P<0.05
8	MS	-		147	P<0.05	-		-	
9	MS	-		212	P<0.05	-		-	
10	MS	-		-		100	P<0.05	-	
11	MS	179	P<0.05	-		-		-	
12	MS	-		-		-		-	
13	MS	-		-		-		-	
14	MS	-		-		-		-	
15	MS	-		-		-		-	
16	MS	-		-		-		-	

Sera and CSF levels of relaxin in MS and controls. No measurable relaxin was detected in either the sera or CSF of the controls. In the MS samples 6 subjects had measurable relaxin. These were divided into 2 cohorts. Cohort 1 consisted of 3 subjects who had measurable levels of relaxin 1 in sera (179 ng/ml) or CSF (179.5 ng/ml). Cohort 2 consisted of 3 subjects who had measurable levels of relaxin 3 in sera (100 ng/ml) or CSF (6.95 ng/ml). The cohorts were distinct as none of the samples contained both relaxin 1 and 3.

In contrast, the presence of RLN1 in the sera and CSF of some of the subjects with MS is intriguing. Prior to this study RLN1 has not been associated with any function and some have postulated that RLN1 may be a pseudogene (Feijóo-Bandín, 2017). The presence of RLN1 in the sera and CSF of subjects with MS suggests an up-regulation of expression in these subjects. Which would indicate that RLN is not a pseudogene but plays a role in metabolism. Further, presence of RLN1 in the sera of some patients and CSF of others suggests expression in multiple organs.

The identification of mutations in the DNA sequence of RLN1 from subjects with MS is of interest (Garvin and Burns, 2016). These mutations are clustered in the coding region for the binding motif of RLN1. Leading to the hypothesis that RLN1 in subjects with MS may not be fully functional. In this case, the production of RLN2 during the later stages of pregnancy would obviate RLN1. And may play a role in the remission of symptoms during this time. Clearly, more work is needed to characterize the role of RLN1 in MS, if any.

The interest in RLN and MS is further spurred on by its diverse modes of action (Bathgate et al, 2013). The ability of RLN to act as a ligand for the glucocorticoid receptor (Dschietzig, 2004), PPARg (Singh, 2010) and to modulate the immune system are areas of intense research in MS (Frischer et al, 2009).

In conclusion, there is some evidence that RLN1 may not be fully functional in subjects with MS. If so, then decreased RLN1 activity could play a role in the pathogenesis of the disease. The increased expression of RLN2 during pregnancy would obviate the need for RLN1. This may play a role in the remission of symptoms seen during pregnancy. And raises the possibility of using relaxin as a novel therapeutic agent in subjects with MS.

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Conflicts of Interest

The author is employed by BVBiomed Ltd.

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