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STUDY TITLE:	Measurement of Relaxin in the Serum and Cerebrospinal Fluid of Subjects With and Without the Relapsing Form of Multiple Sclerosis
PRINCIPAL INVESTIGATOR:	Stanley Cohan, M.D., Ph.D.
INSTITUTION:	Providence Brain and Spine Institute Providence Health & Services
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INTRODUCTION:

MS is an auto-immune disease of the central nervous system (CNS), with more than 400,000 cases in the United States. It is the cause of severe disability in 75-85% of subjects developing the disease[1]. Recognition that MS is largely an auto-reactive lymphocyte-driven disease has led to the development of therapeutic strategies which have resulted in decreased risk of clinical relapses, reduction in progression of lesion burden on MRI scans, and, in some cases, reduced disability progression over the short term, generally for 2 to 3 years[2-5]. It is not known whether there is significant therapy-induced long term reduction in disability progression risk, although some recent data suggests that relapse reduction, particularly early in the disease course, may be associated with less risk of disability progression[6].

The current FDA approved therapeutic agents pose significant difficulties. The first-line medications, interferon beta, glatiramer acetate, teriflunomide, monomethyl/dimethyl fumarate esters and diroximel, provide modest efficacy[2-5], and despite excellent safety profiles, have side effects that are a major obstacle to compliance by many patients. The second line medications, natalizumab, fingolimod, ozanimod, siponimod, ponesimod, ocrelizumab and ofatumumab, have greater efficacy, and are better-tolerated from the perspective of side effects, but carry much greater risk of serious adverse events[7-10]. The need for new therapeutic agents continues, with emphasis being placed not only upon improved efficacy and tolerability, but also greater safety.

It is well-known that MS clinical disease activity abates during pregnancy, although the mechanism(s) of these changes are not understood. Investigations of estrogens and progesterone have not provided consistent data supporting their potential role in MS protection during pregnancy. Relaxin (RLX), a peptide member of the insulin superfamily, is dramatically up-regulated and plays an important role in pregnancy. It also is an immune regulator and may contribute to the auto-tolerance that seems to develop during pregnancy in females with MS. The data that supports a potential role for RLX as an auto-immune mediator, and a potential therapeutic agent in multiple sclerosis, is presented in the paragraphs that follow.

Perioxisomal Proliferator-activated Receptor-Gamma (PPARG):

PPARG is part of a superfamily of nuclear hormone, ligand-activated transcription factors, that are receptors with DNA-linked and C-terminal ligand-linked domains [11]. Ligand activation of these receptors results in reduced pro-inflammatory and increased anti-inflammatory gene expression. PPARG is also constitutionally expressed on T-cell lymphocytes and macrophages in the periphery[12], on

astrocytes and microglia within the CNS, and may not only regulate systemic immune responses, but also regulate inflammatory responses within the CNS[13]. PPARG agonists reduce T-cell proliferation [14], inhibit production of the pro-inflammatory cytokines IL-1B, IL-2, IL-6, IL-12 [15], interferon gamma (IFNG) and TNFa [14], and promote T-cell apoptosis [14, 16]. PPARG ligand agonists also inhibit expression of IFNG-stimulated pro-inflammatory chemokine production by endothelial cells[17], thus potentially inhibiting inflammatory cell trafficking.

Thiazolidinedone (TZD) anti-glycemic agents, such as pioglitazone and rosiglitazone, are PPARG agonist ligands that have both antiglycemic and anti-inflammatory properties [16]. Pioglitazone and rosiglitazone inhibit mitogen-stimulated T-cell proliferation, as well as TNFa and IFNG production[14].

In experimental allergic encephalomyelitis (EAE), used as an animal model of MS, TZDs reduce lymphocyte proliferation and infiltration into the CNS[18], block IL-12-induced TH0 differentiation into pro-inflammatory TH1 T cells[19], reduce gene expression for IL-1B and IL-6, reduce production of TNFa and IFNG, and reduce clinical disease expression [14].

PPARG knockout mice are more susceptible to developing EAE[15], and PPARG antagonists block the neuroprotective effects of the TZDs in EAE[20]. In limited studies in MS patients, PPARG expression is reduced in peripheral immune cells and these cells have reduced responsiveness to PPARG agonists[14].

Relaxin biology overview:
Relaxins (RLX) are members of the insulin-relaxin peptide family[21, 22], that do not bind to insulin receptors and have no effects on glucose metabolism [23]. RLXs have been shown to regulate uterine contraction and cervical growth and softening during pregnancy[24], are vasodilators and promoters of angiogenesis and may be protective in ischemic cardiovascular disease [25]. RLX may also affect immune function, promoting anti-inflammatory mechanisms. There are 3 known isoforms of RLX, RLX 2 being the most abundant circulating form in humans [24]. The gene responsible for RLX2 production is on chromosome 9[26], and is produced primarily in the corpus luteum of females, and heart, lung and kidney in males [27]. RLX acting via its receptor RXFP-1, increases cAMP production[22] and nitric oxide production via NO synthetase[25].

Relaxin as an immune-modulating/regulating agent:
RLX increases PPARG transcription activity via the receptor Relaxin Family Petide-1 (RXFP-1)[21, 23, 28], without increasing PPARG mRNA or PPARG protein levels[28]. RLX independently promotes PPARG via RXRP-1. In addition to its direct PPARG promoting effects, it also increases responsiveness PPARG to TZDs such as rosiglitazone[28]. Given the known anti-inflammatory effects of PPARG promotion, this is one possible mechanism by which RLX impacts immune function. A second mechanism likely involves promotion of the glucocorticoid receptor (GCR) gene[29]. Although found in cell nucleus, exogenous RLX can also

enter the cell nucleus and up-regulate GCR gene, leading to increased production of GCR protein, whereas corticosteroids down-regulate the production of GCR protein, thus creating a negative feedback that can be opposed by RLX competition for the GCR gene site. RLX blocks mitogen-induced production of IL-1, IL-6 and TNFa by macrophages via GCR gene activation, which can be blocked by the GCR inhibitor RU-486[29].

Despite laboratory data demonstrating the potential for RLX as an immune regulating molecule via PPARG[28] and GCR[29] pathways, and its safety in the limited settings in which it has been given to humans for treatment of systemic sclerosis[30] and ischemic heart disease [31], there have been no published preclinical or clinical studies addressing potential efficacy of RLX in inflammatory CNS disease, and only one presented abstract in which it was reported that patients diagnosed with MS had higher circulating levels of RLX, and reduced binding affinity to RXFP-1, when compared to normal controls, suggesting that the increase in circulating RLX was the result of loss of RLX-RXFP-1 regulatory feedback mechanisms. Further analysis revealed that there was structural abnormality in the MS patients' RLX due to a mutation in the B- chain binding cassette of RX (Garvin and Burns, personal communication).

RESEARCH STRATEGY

1. Because of the potential benefits of using a human molecule, with an encouraging safety profile, capable of regulating immune responses, we intend, in the future, to develop a therapeutic trial to assess the efficacy and safety of RLX in the treatment of patients with the relapsing form of MS. However there is no information available on such fundamental questions as what serum and CSF levels of RLX are observed in patients with active versus stable relapsing MS, how these values compare to that found in human volunteers without MS or other inflammatory diseases, and whether RLX functions normally in subjects with active or stable MS. It is for this latter reason that we are proposing research to obtain baseline information on serum and CSF RLX levels in patients with MS, as well as to further study RXFP-1 receptor binding affinity for RLX in patients with active and clinically stable MS. Fortunately there are reliable, commercially available SIMOA assays to measure RLX serum and CSF concentrations and available cell-based RXFP-1 affinity assay systems.

PROTOCOL

Subjects:

All subjects will be age 18 to 60, non-pregnant females, since 65 to 80% of MS patients are female, and there are known gender differences in circulating RLX levels. Patients with known history of diabetes will also be excluded.

There will be 3 groups of subjects:

Group 1 will consist of 10 subjects with suspected MS, who have had a clinical attack within the last 12 weeks, have at least one gadolinium-enhancing lesion on brain or spinal cord MRI taken within the prior 4 weeks, and for which they have not received any immunomodulating or immunosupressant medication. Because a subject in this group may have had a lumbar puncture in an emergency dept or in-patient setting for diagnostic purposes, prior to the investigators becoming aware of the patient, consent may be obtained post LP in this circumstance, and the analyses performed on residual CSF and serum, provided it had been handled in a manner not to interfere with the validity of the analyses. These subjects will also have had a urine pregnancy test performed prior to any relaxin analyses being performed on their samples.

Group 2 will consist of 10 subjects with clinically stable definite MS, with no evidence of clinical relapse for at least the past 12 weeks, and have no gadolinium enhancing lesions on MRI in the prior 12 weeks. These subjects will fulfill the Revised (2017) McDonald's Criteria for the Diagnosis of MS.

Group 3 will consist of 10 subjects without evidence of inflammatory systemic or inflammatory CNS disease, who require CSF removal for some other cause, such treatment of benign intracranial hypertension or as part of the procedure for insertion of an intrathecal medication delivery system.

Study Procedures:
All subjects will sign an IRB-approved informed consent prior to undergoing any study procedures. For patients with reproductive potential, urine sample will be collected and processed with the priority of STAT for qualitative pregnancy testing. The results of the STAT urine pregnancy test will be reviewed by research staff and routed to the study physician. The LP will not proceed unless a negative urine pregnancy test is confirmed. The one exception to this will be in Group 1 patients who had already had LP performed on an emergency basis and in whom consent to analyze residual CSF and serum was obtained. In this latter instance pregnancy test result will be obtained before any relaxin analysis is performed. Every patient will provide blood sample within 24 hours before, or 24 hours after the LP. Whole blood (8-ml) will be processed and serum (5-mL) collected for study analysis. In the case of CSF removal for suspected MS (Group 1), lumbar puncture and CSF removal is not a departure from standard of diagnosis/care, however an additional 5 mL of fluid will be collected for the purpose of this study during the standard procedure. In the case of the non-MS controls (Group 3), CSF removal is a routine part of their therapy and no additional fluid will be removed (5 mL of their fluid will be used for this study). Lumbar puncture and CSF removal is not a routine part of the diagnosis or care of patients with stable MS (Group 2), and thus their informed consent would reflect the fact that they are volunteering for a clinically unnecessary invasive procedure. No patient in Group 2, with clinically stable MS, will be responsible for the costs of performing the lumbar puncture, whereas the patients

in groups 1 and 3 would be having lumbar puncture in the routine course of the management of their disease, and thus the cost of the procedure should be borne by their insurer. The total volume of CSF for Group 2 is also 5 mL.

All study samples will be stored frozen before batch transport to Quanterix for testing.

Approximately 24 hours after the LP procedure research staff will contact the patient by phone to discuss any symptoms or serious adverse events. The outcome of the phone call will be routed to the physician and the patient will be encouraged to contact the study doctor with any additional questions or concerns. All SAEs will be reported the Institutional Review Board per institutional policy and guidelines.

Analysis:
Descriptive statistics will be applied to each variable show the distribution of the data. T-tests or nonparametric tests, depending on the type and distribution of the data, will be performed to compare the differences between groups.

Results:
Patients may be informed of the results of their study testing only after the completion and analysis of all study data.

CONFIDENTIALITY:
Each volunteer subject will be assigned a unique identification number, and this will be used to identify all samples sent to the diagnostic laboratory. The subjects' names, or any other personal identification information will never be available to the diagnostic laboratory or its members.

The results of the data will be returned to the primary investigators for analysis, identified by unique code only, and only after the data has been received will the group that each sample belongs to be identified. The sole individual who knows both personal identification and code numbers will always be blinded to the analytic results and will not share the identity of any subject with the other investigators.

ASSAYS
RLX 1 is measured in serum and CSF by DuoSet (R & D Systems # DY3257) sandwich ELISA. This method is RLX 1 specific and does not cross react with RLX 2, The range of sensitivity of the assay is from 100-10000 pg/mL., and is linear within this range.

RLX 2 is measured in serum and CSF by Quantikine (R & D Systems # DRL 200) sandwich ELISA. This method is RLX 2 specific and does not cross react with RLX 1. The range of sensitivity of the assay has been demonstrated to be 7.8 to 500 pg/mL and is linear within this range.

RLX-RXFP-1 affinity is measured as a function of cAMP production in human monocyte THP-1 cells expressing RXFP-1, following incubation with RLX 1 or RLX 2, both of which act as agonist ligands for this G protein-coupled receptor.

The cAMP levels will also be determined by a commercially available cAMP assay (Flash Plate RAI 125 cAMP assay-Perkin Elmer) in the presence and absence RX neutralizing antibodies. All RLX assays will be performed at Quanterix Labs, Lexington MA. .

These analyses will provide us with quantitative data re: baseline RLXlevels in serum and CSF in subjects with and without known inflammatory CNS disease, as well as alterations in RLX receptor affinity that may occur, and determine whether there are differences in serum/CSF RLX levels and RLX receptor affinities during acute inflammatory and stable states in patients with the relapsing form of MS.

INSTITUTIONAL SUPPORT

The Providence Brain and Spine Institute's clinical research team consists of a manager, two full-time research nurses, five full-time clinical research coordinators (CRC), and 3 part-time clinical research assistants. In 2012, the team supported more than 30 active clinical trials in Alzheimer's disease, ALS, multiple sclerosis, Parkinson's disease, and stroke. The research staff has the experience and skills in conducting cooperative group trials, industry-sponsored trials, and investigator-initiated studies. For each trial, the research staff works closely with the investigators to coordinate study activities, collect clinical research data, track patient and study status, and perform quality assurance activities regularly. The research team is familiar with clinical trials that require multiple specimen collections, diagnostic testing, and neurological and other clinical assessments. The MS Center has 4 full-time providers (5 MS neurologist and 1 NP) and a team of clinical support staff that take care of close to 3,000 MS patients. The group has successfully collaborated with industry and federally-funded sponsors, clinical research organizations, investigators, and ancillary services facilities on more than 30 MS studies in the past 5 years.

For the performance of the serum and CSF RLX and Sema4A levels and RXFP-1 RLX receptor affinity analyses, commercial and university laboratories will be employed as noted in the protocol.

REFERENCES

1. Weinshenker BG. Databases in MS research: pitfalls and promises. Mult Scler 1999;5:206-211.
2. Jacobs LD, Cookfair DL, Rudick RA, et al. Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. The Multiple Sclerosis Collaborative Research Group (MSCRG). Annals of neurology 1996;39:285-294.
3. Comi G, Barkhof F, Durelli L, et al. Early treatment of multiple sclerosis with Rebif (recombinant human interferon beta): design of the study. Mult Scler 1995;1 Suppl 1:S24-27.
4. Johnson KP, Brooks BR, Cohen JA, et al. Copolymer 1 reduces relapse rate and improves disability in relapsing-remitting multiple sclerosis: results of a phase III multicenter, double-blind placebo-controlled trial. The Copolymer 1 Multiple Sclerosis Study Group. Neurology 1995;45:1268-1276.
5. Randomised double-blind placebo-controlled study of interferon beta-1a in relapsing/remitting multiple sclerosis. PRISMS (Prevention of Relapses and Disability by Interferon beta-1a Subcutaneously in Multiple Sclerosis) Study Group. Lancet 1998;352:1498-1504.
6. Bermel RA, Weinstock-Guttman B, Bourdette D, Foulds P, You X, Rudick RA. Intramuscular interferon beta-1a therapy in patients with relapsing-remitting multiple sclerosis: a 15-year follow-up study. Mult Scler 2010;16:588-596.
7. Bourdette D, Gilden D. Fingolimod and multiple sclerosis: four cautionary tales. Neurology 2012;79:1942-1943.
8. Rudick R, Polman C, Clifford D, Miller D, Steinman L. Natalizumab: bench to bedside and beyond. JAMA neurology 2013;70:172-182.
9. Kappos L, Radue EW, O'Connor P, et al. A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. The New England journal of medicine 2010;362:387-401.
10. O'Connor P, Wolinsky JS, Confavreux C, et al. Randomized trial of oral teriflunomide for relapsing multiple sclerosis. The New England journal of medicine 2011;365:1293-1303.
11. Blumberg B, Evans RM. Orphan nuclear receptors--new ligands and new possibilities. Genes & development 1998;12:3149-3155.
12. Harris SG, Phipps RP. The nuclear receptor PPAR gamma is expressed by mouse T lymphocytes and PPAR gamma agonists induce apoptosis. European journal of immunology 2001;31:1098-1105.
13. Storer PD, Xu J, Chavis J, Drew PD. Peroxisome proliferator-activated receptor-gamma agonists inhibit the activation of microglia and astrocytes: implications for multiple sclerosis. Journal of neuroimmunology 2005;161:113-122.
14. Schmidt S, Moric E, Schmidt M, Sastre M, Feinstein DL, Heneka MT. Anti-inflammatory and antiproliferative actions of PPAR-gamma agonists on T lymphocytes derived from MS patients. Journal of leukocyte biology 2004;75:478-485.

15. Bright JJ, Musuro BF, Du C, Sriram S. Expression of IL-12 in CNS and lymphoid organs of mice with experimental allergic encephalitis. Journal of neuroimmunology 1998;82:22-30.
16. Bright JJ, Kanakasabai S, Chearwae W, Chakraborty S. PPAR Regulation of Inflammatory Signaling in CNS Diseases. PPAR research 2008;2008:658520.
17. Marx N, Mach F, Sauty A, et al. Peroxisome proliferator-activated receptor-gamma activators inhibit IFN-gamma-induced expression of the T cell-active CXC chemokines IP-10, Mig, and I-TAC in human endothelial cells. J Immunol 2000;164:6503-6508.
18. Niino M, Iwabuchi K, Kikuchi S, et al. Amelioration of experimental autoimmune encephalomyelitis in C57BL/6 mice by an agonist of peroxisome proliferator-activated receptor-gamma. Journal of neuroimmunology 2001;116:40-48.
19. Shevach EM, Chang JT, Segal BM. The critical role of IL-12 and the IL-12R beta 2 subunit in the generation of pathogenic autoreactive Th1 cells. Springer seminars in immunopathology 1999;21:249-262.
20. Raikwar HP, Muthian G, Rajasingh J, Johnson CN, Bright JJ. PPARgamma antagonists reverse the inhibition of neural antigen-specific Th1 response and experimental allergic encephalomyelitis by Ciglitazone and 15-deoxy-Delta12,14-prostaglandin J2. Journal of neuroimmunology 2006;178:76-86.
21. Bathgate RA, Ivell R, Sanborn BM, Sherwood OD, Summers RJ. International Union of Pharmacology LVII: recommendations for the nomenclature of receptors for relaxin family peptides. Pharmacological reviews 2006;58:7-31.
22. Halls ML, Bathgate RA, Summers RJ. Relaxin family peptide receptors RXFP1 and RXFP2 modulate cAMP signaling by distinct mechanisms. Molecular pharmacology 2006;70:214-226.
23. Hsu SY, Nakabayashi K, Nishi S, et al. Activation of orphan receptors by the hormone relaxin. Science 2002;295:671-674.
24. Sherwood OD. Relaxin's physiological roles and other diverse actions. Endocrine reviews 2004;25:205-234.
25. Bani D. Relaxin as a natural agent for vascular health. Vascular health and risk management 2008;4:515-524.
26. Crawford RJ, Hudson P, Shine J, Niall HD, Eddy RL, Shows TB. Two human relaxin genes are on chromosome 9. The EMBO journal 1984;3:2341-2345.
27. Hayes ES. Biology of primate relaxin: a paracrine signal in early pregnancy? Reproductive biology and endocrinology : RB&E 2004;2:36.
28. Singh S, Bennett RG. Relaxin signaling activates peroxisome proliferator-activated receptor gamma. Molecular and cellular endocrinology 2010;315:239-245.
29. Dschietzig T, Bartsch C, Stangl V, Baumann G, Stangl K. Identification of the pregnancy hormone relaxin as glucocorticoid receptor agonist. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2004;18:1536-1538.

30. Seibold JR, Clements PJ, Furst DE, et al. Safety and pharmacokinetics of recombinant human relaxin in systemic sclerosis. The Journal of rheumatology 1998;25:302-307.
31. Dschietzig T, Teichman S, Unemori E, et al. Intravenous recombinant human relaxin in compensated heart failure: a safety, tolerability, and pharmacodynamic trial. Journal of cardiac failure 2009;15:182-190.