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RECOMBINANT T-CELL RECEPTOR LIGAND RTL1000 LIMITS INFLAMMATION AND DECREASES INFARCT SIZE AFTER EXPERIMENTAL ISCHEMIC STROKE IN MIDDLE-AGED MICE

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Abstract—We have previously demonstrated that recombinant T-cell receptor ligand 1000 (RTL1000) reduces infarct size and improves long-term functional recovery after experimental stroke in young transgenic mice expressing human leukocyte antigen DR2 (DR2-Tg). In this study, we determined the effect of RTL1000 on infarct size in 12-month-old middle-aged DR2-Tg mice, and investigated its mechanism of action. Twelve-month-old male DR2-Tg mice underwent 60 min of intraluminal reversible middle cerebral artery occlusion (MCAO). Vehicle or RTL1000 was injected 4, 24, 48 and 72 h after MCAO. Cortical, striatal and total hemispheric infarcts were measured 96 h after stroke. Spleen and brain tissues were collected 96 h after stroke for immunological analysis. Our data showed that RTL1000 significantly reduced infarct size 96 h after MCAO in middle-aged male DR2-Tg mice. RTL1000 decreased the number of activated monocytes/microglia cells ($CD11b^+CD45^{hi}$) and $CD3^+$ T cells in the ischemic hemisphere. RTL1000 also reduced the percentage of total T cells and inflammatory neutrophils in the spleen. These findings suggest that RTL1000 protects against ischemic stroke in middle-aged male mice by limiting post-ischemic inflammation.

Key words: ischemic stroke, immunotherapy, recombinant T-cell receptor ligand, HLA-DR2 transgenic mice.

INTRODUCTION

Experimental stroke induces rapid activation of the peripheral immune system, which contributes to the brain's inflammatory response to stroke (Nilupul Perera et al., 2006; Dirnagl et al., 2007; Gee et al., 2007; Muir et al., 2007). The migration of monocytes, neutrophils and T cells into the brain with the breakdown of the blood–brain barrier contributes to the further activation of resident microglial cells and the expansion of brain tissue infarction. Among these cells, T cells are found in the brain within hours after experimental stroke, which may play a significant role in exacerbating ischemic injury. T- and B-cell-deficient mice sustain smaller lesion size and reduced inflammation after experimental stroke (Hurn et al., 2007), with $CD4^+$ and $CD8^+$ T lymphocytes playing a particularly important role in the inflammatory and thrombogenic response associated with experimental stroke by promoting an autoaggressive response to brain antigens (Yilmaz et al., 2006). It is believed that myelin-reactive antigens leak out of the brain with the breakdown of the blood–brain barrier, which is recognized by the immune system as a foreign antigen, leading to the recruitment of T cells into the brain. These conclusions are supported by the findings of increased influx of myelin oligodendrocyte glycoprotein (MOG)-specific T cells into the brain and of reduced infarct size after stroke by nasal vaccination with a MOG peptide (Frenkel et al., 2003).

Recombinant T cell ligands (RTLs) are a class of partial major histocompatibility complex (MHC) class II molecules comprised of covalently linked α1 and β1 chains that are tethered to a MOG peptide (Burrows et al., 1999; Wang et al., 2003; Vandenbark et al., 2003). We have previously demonstrated that RTL551, a mouse MHC moiety ($I\text{-}A^b$) coupled to mouse myelin peptide (mMOG-35-55), reduces infarct size in 3-month-old young adult C57BL/6 mice (Subramanian et al., 2009; Dziennis et al., 2011). The action mechanism involves in selective modulation of auto-aggressive CD^{4+} T cells by delivering partial agonist signals through the T cell receptor (TCR), and further inhibition of the accumulation of other inflammatory cells, particularly macrophages/activated microglial cells and dendritic

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Abbreviations: CBF, cortical blood flow; CCA, common carotid artery; ECA, external carotid artery; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MHC, major histocompatibility complex; MOG, myelin oligodendrocyte glycoprotein; RTL1000, recombinant T-cell receptor ligand 1000; RTLs, recombinant T cell ligands; SAH, subarachnoid hemorrhage; STAIR, Stroke Therapy Academic Industry Roundtable; TCR, T cell receptor.

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cells, a kind of antigen presenting cells that assist with activation of T cells in the brain.

We have previously found that RTL treatment is antigen-specific and MHC-specific. Our data show that RTL553, which has the same MHC moiety as RTL551 but is linked to a non-neuroantigen peptide (I-Ea-52-68), had no effect on infarct size in C57BL/6 mice. Similarly, RTL treatment with RTL342M, which has the same mMOG-35-55 peptide as RTL551 but a different MHC II moiety (HLA-DR2), failed to reduce infarct size (Dziennis et al., 2011). These findings indicate that RTL551 may not work in patients with stroke considering species-differences (murine vs. human) in antigens and MHC II molecules. To determine if a RTL strategy would work against human stroke, we determined the efficacy of humanized recombinant T-cell receptor ligand 1000 (RTL1000), which contains a human MHC moiety (HLA-DR2) covalently linked to a human myelin peptide (hMOG-35-55) in experimental stroke in humanized DR2-Tg mice which expresses human TCR (Subramanian et al., 2009; Zhu et al., 2014a). We found that RTL1000 indeed protects against ischemic injury in young male DR2-Tg mice. Behavioral testing showed that RTL1000 improves long-term cognitive function 28 days after stroke (Zhu et al., 2014a). A similar effect has also been demonstrated in young female DR2-Tg mice (Pan et al., 2014). We also confirmed that combining RTL1000 with t-PA does not alter its ability to reduce infarct in experimental ischemic stroke (Zhu et al., 2014b).

It's well known that ischemic stroke risk increases with age, and stroke is most common in the aging population. Unfortunately, most animal studies, including our own previous study using RTL1000 were conducted in healthy young mice. The Stroke Therapy Academic Industry Roundtable (STAIR) has identified age as an important factor to be considered in developing therapeutic agents for the treatment of stroke (Stroke therapy academic industry roundtable, 1999). In order to meet the preclinical STAIR criteria (Stroke therapy academic industry roundtable, 1999; Fisher et al., 2009), and to move our research findings closer to clinical practice, in the present study, we evaluated the efficacy of RTL1000 in protecting against ischemic in middle-aged (12-month-old) humanized DR2-Tg mice. Furthermore, successful preclinical evaluation requires target validation to ensure outcomes are indeed linked to the purported mechanism of action of the compound (Feuerstein et al., 2008). Therefore, in the current study, we additionally validated that RTL1000 indeed reduces post-ischemic inflammation by limiting the infiltration of inflammatory cells into the brain in middle-aged mice.

EXPERIMENTAL PROCEDURES

Ethics statement

Animal experiments were conducted in accordance with National Institutes of Health guidelines for the use of experimental animals. The protocols were approved by the Animal Care and Use Committee at the Oregon Health & Science University and the Portland Veteran Affairs Medical Center. All efforts were made to minimize the number of animals used and their suffering.

Animals and experimental groups

Studies were performed on male HLA-DRB1*1502 transgenic (DR2-Tg) mice (produced at the Portland VA Medical Center with foundation breeders provided by Dr. Chella David (Gonzalez-Gay et al., 1996)) aged 12 months (ranging from 52 to 54 weeks) and weighing 25.6–39.5 g (a total of $n = 47$). Thirty-three mice were used for infarct size analysis (16 in the RTL1000 group and 17 in the vehicle group) and splenocyte number counting and survival assay. Separate groups of seven mice per group were used for the analysis of cell populations in the brain and spleen assay by flow cytometry. Mice were randomly assigned to either RTL1000 or vehicle groups, and investigators were blinded to treatment groups during surgery and tissue analysis.

RTL 1000 production and purification

RTL molecules consist of the α1 and β1 domains of the MHC II molecule expressed as a single polypeptide with or without antigenic amino terminal extensions (Burrows et al., 1999; Huan et al., 2005). RTL1000 is a HLA-DRB1*1502 (DR2) molecule linked to human MOG^{-35-55} peptide (MEVGWYRPPFSRVVHLYRNGK) (Subramanian et al., 2009). RTL1000 was constructed *de novo* or by sequential site-directed mutagenesis of previous constructs. Protein purification was performed with a 30- to 40-mg yield of purified protein per liter of bacterial cell culture.

RTL1000 treatment

Mice were randomized to injections of 100 μL of either RTL1000 (1 μg/μL) or vehicle (5% dextrose in Tris–HCl, pH 8.5) 4 h after stroke by subcutaneous (S.C.) injection, which was followed by 3 injections of the same volume and concentration at 24, 48, and 72 h after middle cerebral artery occlusion (MCAO). The dose and injection protocol of RTL1000 was established in our previous studies (Subramanian et al., 2009; Zhu et al., 2014a,b).

Reversible MCAO

Reversible MCAO was induced via the intraluminal filament technique as described previously with slight modifications (Zhu et al., 2010). Mice were anesthetized with isoflurane (5% induction; 1.2% maintenance) using a mask connected with a vaporizer (Isotec 4; Cyprane, England) throughout surgery and during a 60-min vascular occlusion until filament withdrawal and initiation of reperfusion. Rectal temperature was monitored and maintained at 36.5 ± 0.5 °C throughout surgery with a warm water pad and a heating lamp. Cortical blood flow (CBF) was monitored by laser-Doppler flowmetry (LDF; Model DRT4, Moor Instruments Ltd., Oxford, England). The right lateral common carotid artery (CCA) was exposed and temporarily ligated. The right external carotid artery (ECA) was ligated and cauterized. Ipsilateral MCAO