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Tissue plasminogen activator involvement in experimental autoimmune myasthenia gravis: Aggravation and therapeutic potential

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ABSTRACT

Tissue plasminogen activator (tPA), a component of the PA/plasmin system, is elevated in inflammatory areas and plays a role in inflammatory neurological disorders. In the present study we explored the involvement of tPA and the potential immunomodulatory activity of tPA in experimental autoimmune myasthenia gravis (EAMG). Mice deficient in tPA ($tPA^{-/-}$) present with a markedly more severe disease than wild type EAMG mice. In an attempt to treat EAMG with an 18aa peptide derived from the PA system inhibitor (PAI-1), designed to tether out the endogenous inhibitor, a significant suppression of disease severity was demonstrated. The more severe disease in $tPA^{-/-}$ mice was accompanied by a higher level of anti-AChR antibodies and increased expression of B-cell markers. In view of the essential role of B-cell activating factor (BAFF) in B-cell maturation, the expression of BAFF family components was tested. An increase in BAFF and BAFF receptor was observed in EAMG $tPA^{-/-}$ mice, whereas BCMA expression was reduced, consistent with the increased level of pathogenic antibodies and the more severe disease. Given the importance of T regulatory cells (Tregs) in EAMG, they were evaluated and their number was reduced in $tPA^{-/-}$ mice, in which EAMG was aggravated, whereas following PAI-1dp treatment, Tregs were replenished and the disease was ameliorated. The results show the involvement of tPA in EAMG, implying a protective role for tPA in EAMG pathogenesis. The amelioration of EAMG by PAI-1dp treatment suggests that the PA system may be considered a potential site for therapeutic intervention in neuroimmune diseases.

1. Introduction

Extracellular proteases such as plasminogen activators (PAs) and matrix metalloproteinases modulate cell-cell and cell-matrix interactions. There is evidence for the involvement of the PA system in both physiological and pathological immune processes [1–4]. The PA system in particular was shown to participate in the function and pathology of the nervous system. Changes in the expression of PA system components have been observed in a variety of neurological disorders such as epilepsy, stoke, brain trauma, Alzheimer's disease, cerebral malaria, HIV-associated leukoencephalopathy, encephalitis and multiple sclerosis. Tissue PA (tPA), a component of the PA/plasmin system, is elevated in inflammatory areas, and was shown to play a role in inflammatory neurological disorders [1,2].

As a member of the serine protease family, tPA participates in the activation of various zymogens and certain growth factors and is involved in the homeostasis of blood coagulation/fibrinolysis and matrix regulation. However, recent studies show that tPA also acts as a profibrotic cytokine that promotes inflammatory processes by triggering receptor-mediated intracellular signaling events. Cao et al. demonstrated the involvement of tPA in macrophage migration during inflammation: In response to acute inflammation, macrophages accumulate at the site of injury, followed by Mac-1-dependent trafficking to the lymphatic system where they present antigens to lymphocytes. This is achieved through interaction with plasminogen activator inhibitor 1(PAI-1) and the lipoprotein receptor (LRP) [5].

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The activity of the PA system components is monitored closely and regulated by serine PAI, of which PAI-1 is most prominent. PAI-1 binds active tPA and forms a tPA-PAI-1 complex, followed by its internalization and intracellular degradation [6]. The role of the PA system has never been studied in myasthenia gravis (MG) or in experimental autoimmune MG (EAMG). Here we explored the involvement of tPA in the development of EAMG, the animal model used for the study of MG.

MG is an antibody-mediated autoimmune disease in which the primary autoantigen is the skeletal muscle acetylcholine receptor (AChR) [7]. The immunopathogenesis of MG involves the production of anti-AChR antibodies whose synthesis is modulated by specific CD4+ T cells [8]. EAMG can be induced in a wide variety of experimental animals by immunization with AChR purified from the electric organs of the electric ray, Torpedo californica (T-AChR) [9,10]. In both MG and EAMG, AChR-specific B cells produce anti-AChR antibodies that bind to the AChR at the neuromuscular junction (NMJ), activate complement, and accelerate AChR destruction, culminating in neuromuscular transmission failure and muscle weakness [11,12]. The hallmark pathological feature of MG and EAMG is destruction of the NMJ preceded by accumulation of IgG and complement deposits at the junction [13].

Development of the typical myasthenic muscle weakness results from a cascade of events involving interactions between various cell types and the activity of many immune mediators such as complement, cytokines, transcription factors, and B-cell maturation factors.

T regulatory cells (Tregs) are involved in immunomodulation of autoimmunity in general. Tregs are essential for the development of autoimmunity, as has been demonstrated in a number of autoimmune diseases and their experimental models, including MG [14] (Tregs defined as $CD4^{+}CD25^{high}$). The overall number of circulating Tregs (defined as $CD4^{+}CD25^{+}FoxP3^{+}$) in untreated MG patients was found to be lower than in healthy controls [15] and with impaired function in the thymus [16]. In addition, EAMG animals had lower numbers of Tregs with impaired functionality and administration of ex-vivo generated Tregs to myasthenic rats inhibited disease progression [17,18].

B cell activating factor (BAFF), a member of the TNF superfamily, is a potent survival factor for B-cells, and plays an essential role in the maintenance and maturation of peripheral B cells [19]. When overexpressed, BAFF protects B cells from apoptosis, thereby contributing to autoimmunity [19]. Elevated levels of BAFF have been observed in the serum and target organs of MG patients, as well as in other autoimmune diseases [20].

In the present study we induced EAMG in tPA knockout (ko) mice and show that this deficiency results in a markedly more severe disease, accompanied by a reduction in Treg number and an increase in BAFF expression. Furthermore, treatment of EAMG mice with a PAI-1-derived peptide, (PAI-1-dp) suppressed further development of EAMG. Thus, our results support the importance of the tPA in EAMG pathogenesis and point to a potential site for therapeutic intervention.

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from Harlan Laboratories (Rehovot, Israel originated from the Jackson laboratories) and housed under specific pathogen-free conditions in the animal facility of the Hebrew University Medical School, in accordance with NIH guidelines for the care and use of laboratory animals. ko mice deficient for tPA against a C57BL/6 background were purchased from Jackson Laboratories (Jackson Immunoresearch Laboratories, West Grove, PA, USA), bred in the animal facility of The Hebrew University Medical School and housed under specific pathogen-free conditions.

2.2. Preparation of T-AChR

T-AChR was purified from the electric organ of Torpedo californica as previously described [21]. In brief, membrane fragments were solubilized with 1% Triton x-100 followed by affinity chromatography on Naja naja Siamensis neurotoxin-Sepharose resin. The bound receptor was eluted with carbamylcholine. Its specific activity was 3000–4500 pmolα-bungarotoxin (αBgT) binding sites/mg protein.

2.3. EAMG induction and clinical evaluation

Purified T-AChR (25 μg and 5 mg/ml of M. tuberculosis H37Ra, (Difco, Detroit MI), emulsified in complete Freund's adjuvant) was subcutaneously (s.c.) injected into the hind footpads of 6–8-weeks-old wild type (wt) C57BL mice and $tPA^{-/-}$ C57BL mice. A booster injection containing the same amounts was administered after 30 days. The animals were weighed and inspected weekly during the first month and daily after the booster injection to evaluate muscle weakness. The clinical status was graded as follows: (0) no weakness or fatigue, (1) mildly decreased activity, weak grip with fatigue, weight loss >3% body weight in one week; (2) moderate weakness accompanied by weak grip, 5–10%, weight loss (3) moderate-severe weakness, hunched back posture at rest, head down and forelimb digit flexed, tremulous ambulation, 10% weight loss; and (4) severe general weakness, weak grip, weight loss >10%.

2.4. PAI-1-dp treatment

An 18aa peptide, Ac-RMAPEEIIMDRPFLYVVR-amide, derived from the PAI-1 protein (PAI-1-dp) (previously described in Ref. [22]), was used for treatment of the EAMG-induced mice, which were compared with control placebo-treated mice. PAI-1-dp was injected i.p. twice daily at 0.5 mg/kg, starting three weeks after disease induction (before the onset of clinical symptoms) for seven consecutive days. This treatment protocol was adapted from our previous experiments in experimental autoimmune encephalomyelitis (EAE) [23]. Mice were followed clinically as described above.

2.5. Anti-AChR Ab determination

Sera from EAMG animals were assayed by direct radioimmunoassay for Rat-AChR [24], measured 60–90 days after disease induction, when the experiments were terminated.

2.6. Flow cytometry analysis

For leukocyte surface marker determination, pooled spleen cells were obtained from wt and ko mice (as described in the mouse lymphocyte proliferation assay). Cell suspensions were prepared as described previously [25]. For immune phenotyping, the following Abs were used: anti-CD11b-PE, anti-CD11c-PE, anti-CD19-FITC, anti-CD45R-FITC – all from eBioscience San Diego. CA. Stained cells were counted by FACS (Beckman Coulter FC500).

Regulatory T cells were stained using a regulatory T cell staining kit (w/PE FoxP3 FJK-16s, FITC CD4, APC CD25) (eBioscience) FOXP3 FJK-16Abs were used for intracellular staining for FOXP3 expression.