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Apotransferrin inhibits interleukin-2 expression and protects mice from experimental autoimmune encephalomyelitis

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ABSTRACT

Transferrin (Tf) has a major role in T cell activation and proliferation. Here, we investigated whether Tf exerts immunomodulatory effects on T cells and in development of T-cell driven experimental autoimmune encephalomyelitis (EAE). While treatment of concanavalin A-stimulated splenocytes with apotransferrin (ApoTf) did not affect release of IL-1β, TNF, IFN-γ, IL-17, IL-4, and IL-10, it markedly and dose-dependently down-regulated synthesis of IL-2 in these cells. ApoTf also inhibited IL-2 generation in purified $CD3^+$ T cells and the effect was accompanied with down-regulation of MAPK p44/42 and NFκB signaling. Despite impeded IL-2 release, proliferation of splenocytes was not inhibited by ApoTf. Importantly, ApoTf ameliorated EAE in mice and significantly reduced *ex vivo* IL-2 production in proteolipid protein-specific lymphocytes. Thus ApoTf may be a promising beneficial agent for multiple sclerosis.

1. Introduction

Transferrin (Tf) is a multi-functional glycoprotein with a major role in iron transport and metabolism maintaining systemic iron homeostasis (Gkouvatsos et al., 2012; Gomme et al., 2005). Iron is utilized by several proteins as a cofactor for major biological processes, including antimicrobial defence (Ong et al., 2006). However, iron may also harm cells by catalyzing the generation of free radicals and promoting oxidative stress and even apoptosis (van Campenhout et al., 2003). Therefore, iron homeostasis is crucial for normal cell metabolism, and its deficiency or excess is associated with numerous pathologies including autoimmune diseases.

In addition to the control of iron metabolism, Tf may also play a role in the pathogenesis and natural course of autoimmune diseases by regulating differentiation, activation and death of T cells (Macedo and de Sousa, 2008). Tf receptor (TfR, CD71) is induced in T cells with mitogen stimulation, and up-regulated by the influence of IL-2 (Hamilton, 1982; Pelosi-Testa et al., 1988). It was assumed that IL-2 proliferative effects on T cells are partly mediated through up-regulation of TfR (Neckers and Cossman, 1983). Numerous studies provided evidence for clinical relevance of Tf in diseases that are associated with lower plasma Tf concentrations, as well as with Tf gene polymorphisms. These include pathologies with an inflammatory component such as renal ischemia reperfusion injury, diabetes-related complications, stroke, Alzheimer's disease, cancer, atransferrinemia (Gomme et al., 2005). Among the Tf family of proteins, the immunomodulatory capacities of apotransferrin (ApoTf) were previously anticipated by the capacity of this protein to prevent renal ischemia–reperfusion injury (de Vries et al., 2004). In agreement with the potential immunomodulatory action of ApoTf, we have recently demonstrated that patients with newly diagnosed type 1 diabetes (T1DM) manifest significantly lower ApoTf serum levels compared to healthy controls and patients with long-lasting disease (Mangano et al., 2012). In addition, exogenously administered either recombinant or extractive ApoTf reduced the incidence of the disease in well known models of T1DM such as the NOD mouse and the diabetes-prone BB rats. Although there is evidence about the possible role of iron metabolism in pathology of multiple sclerosis (MS) (LeVine and Chakrabarty, 2004), relationship between ApoTf and MS has not been explored so far.

These observations prompted us to evaluate further direct impact of ApoTf on immune system and cytokine secretion *in vitro* and during the development of experimental allergic encephalomyelitis (EAE), a preclinical model of human MS. Our results indicate that ApoTf specifically decreases concanavalin A (ConA)-stimulated IL-2 production via inhibition of MAPK p44/42 and NFκB signaling with no effect on lymphocyte proliferation. Furthermore, amelioration of EAE coincided with reduction

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of IL-2 in autoantigen-specific lymphocytes. This study suggests the possibility of using ApoTf as a novel therapeutic strategy for the treatment of MS and other neuroinflammatory diseases.

2. Materials and methods

2.1. Induction of EAE and experimental design

Six to seven week-old female Swiss Jackson Laboratory (SJL) mice were purchased from Charles River (Calco, Italy). The animals were housed in a controlled environment and provided with ad libitum standard rodent chow and water. Animal care was in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (D.M. 116192), as well as with EEC regulations (O.J. of E.C. L 358/1 12/18/1986). Proteolipid protein (PLP) (139–151) was synthesized by Genemed Synthesis (San Francisco, CA, USA). EAE was induced by immunization of mice with 75 μg PLP (139–151) emulsified in complete Freund's adjuvant (CFA) with 6 mg/ml *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI, USA). Each mouse received subcutaneous injections of 200 μl emulsion divided among two sites draining into the inguinal lymph nodes. Pertussis toxin (Calbiochem, Nottingham, UK) was used as a co-adjuvant, and was administered intraperitoneally (i.p.) at a dose of 200 ng/mouse on day 0 and on day 2. ApoTf was administered i.p. at the dose of 0.1, 1.0 and 2.5 mg/kg daily starting from day 6 post-immunization up to day 30. Control animals were treated with sterile PBS under the same experimental conditions. Animals were monitored daily for clinical signs of EAE. They were assigned one of the following clinical grades by an observer blinded to the treatment: 0, no illness; 1, flaccid tail; 2, moderate paraparesis; 3, severe paraparesis; 4, moribund state; and 5, death. A cumulative clinical score was calculated for each mouse by adding the daily scores from the day of onset (score disease ≥ 1) until the end of the experiment. The duration of disease was calculated by assigning the animal a score of 0 for a clinical score of 0 and 1 for any higher clinical score and summing up the scores for the entire duration of the experiment.

At the end of the experiment, spinal cords were collected for subsequent cell suspension preparation. Lymphocytes infiltrating the spinal cords were isolated after spinal cord homogenization, and the resulting cells centrifuged in a discontinuous gradient of Percoll (35%/70%) (Sigma Aldrich, Milan, Italy). Cells at the interface were collected, washed twice in RPMI 1640 medium supplemented with 10 % fetal calf serum, 2 mM glutamine, and 50 mg/ml of penicillin/streptomycin medium, and counted.

2.2. Cells and cell cultures

For the isolation of cells experimental animals were obtained from the Animal House Facility of the Institute for Biological Research "Sinisa Stankovic", Belgrade. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the institute, and run in accordance to the requirements of the European Union regarding handling of experimental animals. Medium RPMI-1640 supplemented with 1 mM HEPES buffer, 5% fetal calf serum (PAA Chemicals, Pasching, Austria), 1% sodium pyruvate, 2 mM L-glutamine, penicillin/streptomycin (Galenika, Belgrade, Serbia), and 5 μM β-mercaptoethanol was used in cell culture experiments (complete medium). Spleen cells (SPC) were obtained from spleens of C57BL/6, CBA mice, BALB/c mice and NOD mice. To isolate SPC, spleens were mechanically disrupted, passed through 40-μm nylon mesh filter and collected by centrifugation. Red blood cells from single cell suspensions were lysed using RBC Lysis Buffer (eBioscience, San Diego, CA). For the purification of T cells from SPC of C57BL/6 mice, anti-mouse CD3-biotin conjugated antibody (BD Biosciences, San Diego, CA), MACS streptavidin microbeads and MACS separation columns were used according to the manufacturer's instructions (Miltenyi Biotec, Aubum, CA). Apotransferrin (ApoTf) (Calbiochem, La Jolla, CA) and adequate stimulants were added at the beginning of cell cultivation as indicated in the following sections. Human plasma-derived ApoTf was derived by Kedrion (Barga, Italy) from fraction IV-1,4 of the Cohn plasma fractionation process purified by two chromatographic steps.

2.3. ELISA

SPC were seeded at 5×10^6/ml/well in 24-well plates and stimulated with 2.5 μg/ml of ConA (Sigma-Aldrich, St. Louis, MO). $CD3^+$ cells were seeded at 2×10^6/ml/well of 24-well plate and stimulated with plate bound anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) antibodies (eBioscience). After 72 h cell-free cell culture supernatants were collected. Cytokine concentration in the cell culture supernatants was determined by sandwich ELISA using MaxiSorp plates (Nunck, Rochild, Denmark) and anti-mouse paired antibodies according to the manufacturer's instructions. Samples were analyzed in duplicate for murine IL-17, IL-1β, TNF (BD Biosciences), IL-2, IL-10 (eBioscience), IFN-γ, IL-4 (R&D, Minneapolis, MN, USA). The results were calculated using standard curves made on the basis of known concentrations of the appropriate recombinant cytokines.

2.4. ELISPOT

96-well ELISPOT plates (Millipore, Billerica, MA) were coated overnight with anti-IL-2 capture antibody (BD Pharmingen, San Diego, CA) in sterile PBS. The following day, plates were blocked for 1 h with sterile PBS/BSA 0.5% and washed 3 times with sterile PBS. Splenocytes (2×10^5) were added to each well and stimulated overnight at 37 °C in 5% CO_2 in the presence of complete medium (negative control), ConA (positive control), or PLP (20 μg/ml). After washing, wells were incubated with detection antibody for 2 hr at room temperature. The plate bound second antibody was then visualized by adding 5-bromo-4-chloro-30-indolylphosphate p-toluidine salt and nitroblue tetrazolium chloride (chromogen color reagent) to each well (R&D Systems). The plates were then rinsed with distilled water, dried at room temperature and spots counted. Data are presented as the number of IL-2 producing cells per million cells.

2.5. Measurement of proliferation and NO release

For proliferation assay and nitrite measurement, SPC (5×10^5/200 μl/well) or $CD3^+$ cells (2×10^5/200 μl/well) were seeded at in 96-well plates and stimulated for 66 h with ConA (2.5 μg/ml) or with plate bound anti-CD3 and anti-CD28 antibodies, respectively, while the last 18 h of culture in the presence of 37.5 kBq (1 μCi) of [^{3}H]-thymidine (ICN, Costa Mesa, CA). Incorporated radioactivity was measured in a liquid scintillation counter (Beckman Coulter, Fullerton, CA). After 48 h of cultivation, 50 μl of cell culture supernatants were collected and used for determination of nitrites. Nitrite accumulation, as an indirect measure of NO release, was determined in cell culture supernatants using Griess reaction. In brief, cell-free supernatants were mixed with an equal volume of Griess reagent, a 1:1 mixture of 0.1% naphthylethylenediamine dihydrochloride (Sigma-Aldrich) and 1% sulphanilamide (Sigma-Aldrich) in 5% H_3PO_4. The absorbance at 570 nm was determined in a microplate reader (LKB 5060-006; LKB) and compared to a standard curve for $NaNO_2$.

2.6. Analysis by flow cytometry

Lymphocytes were stained with FITC-conjugated anti-CD25 (anti-α-chain of IL-2 receptor) antibodies (BD Biosciences, San Diego, CA) and with PE-conjugated anti-CD4 antibodies (eBioscience, San Diego, CA). Appropriate isotype-matched dye-conjugated antibodies were used as negative control for measurement of background fluorescence. The stained cells were analyzed on Partec flow cytometer (CyFlow® Space, Partec, Münster, Germany).