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B lymphocyte proliferation is suppressed by the opioid growth factor–opioid growth factor receptor axis: Implication for the treatment of autoimmune diseases

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Endogenous opioids are known to repress the incidence and progression of autoimmune diseases. One native opioid peptide, [Met[5]]-enkephalin, termed the opioid gowth factor (OGF), interacts with the OGF receptor (OGFr) to suppress the expression of experimental autoimmune encephalomyelitis. The present study examined the role of the OGF-OGFr axis in the regulation of B lymphocyte proliferation. Murine B lymphocytes were stimulated with lipopolysaccharide. Both OGF and OGFr were present in all B lymphocytes. OGF had a dose-dependent effect on growth, with cell number inhibited by up to 43% at 72 h; no other synthetic or native opioid altered cell proliferation. Exogenous OGF depressed cell number in cultures treated with siRNAs for the classical opioid receptors, MOR (μ), DOR (δ), and KOR (κ), however this peptide had no effect in preparations exposed to siRNA for OGFr. The decrease in cell number by exogenous OGF was dependent on p16 or p21 cyclin-dependent inhibitory kinase pathways. Exposure to the opioid antagonist, naltrexone, did not change cell number from control levels. These results suggest that the OGF-OGFr axis is present and functional in B lymphocytes, but this system is not an autocrine regulator of cell proliferation. Thus, at least exogenous OGF and perhaps endogenous OGF by paracrine/endocrine sources, can be an immunosuppressant. Modulation of the OGF-OGFr axis may be a novel paradigm for the treatment of autoimmune diseases.

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Introduction

Evidence is accumulating that B lymphocytes are a component in many T lymphocyte-mediated diseases (Dörner et al. 2009; Pescovitz et al. 2009; Dornair et al. 2009). B lymphocytes can play a crucial role as antigen-presenting cells (Noorchashm et al. 1999; Rivera et al. 2001), expressing high levels of class II major-histocompatibility-complex antigens (Lapointe et al. 2003; Serrez and Silveira 2003) and generating cryptic peptides to which T lymphocytes are not tolerant (Davidson and Diamond 2001). Morever, B cells provide T cell costimulation, synthesize cytokines, fix complement, and produce autoantibodies (Adamus 2009; Fraussen et al. 2009; Tsirogianni et al. 2009). After encountering antigen and T cell help in follicles of secondary lymphoid organs, mature naive B cells undergo germinal center reactions leading to their clonal expansion and eventually mature into either memory B cells or Ig-secreting plasma cells. Elucidating the mechanism(s) underlying proliferation of B cells has become important in designing B cell targeted therapies (Dörner et al. 2009; Shlomchik 2009; Balague et al. 2009).

Endogenous opioids are immunomodulatory molecules within both the immune system and the brain (Carr et al. 1996; McCarthy et al. 2001; Peterson et al. 1998; Salzet and Tasiemski 2001; Sharp et al. 1998). One native opioid peptide that has received considerable attention in this regard has been [Met[5]]-enkephalin, an endogenous opioid peptide derived from preproenkephalin (Akil et al. 1984; Noda et al. 1982). Originally found to be related only to neural cells and to serve as a neuromodulatory element (Akil et al. 1984), subsequent studies revealed that [Met[5]]-enkephalin is a regulator of neural and non-neural cell proliferation (Zagon et al. 2002). To signify its unique distribution and biological role, this peptide has been termed the opioid growth factor (OGF) (Zagon et al. 2002). OGF action is mediated by the OGF receptor (OGFr). Although the OGF-OGFr system has the same pharmacological properties of opioid peptides that interact with classical opioid receptors (e.g., blockade by naloxone, stereospecificity), OGFr has nucleotide and protein sequences that are entirely different from that of classical opioid receptors (Zagon et al. 2002). OGF-OGFr interactions

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inhibit cell proliferation in a tonically active fashion and rigorously maintain homeostasis of cellular renewal and restorative processes (e.g., wound healing) (Zagon et al. 1997; Wilson et al. 2000; Blebea et al. 2000). The OGF-OGFr axis does not alter differentiation (Zagon and McLaughlin 2005) or migration (Zagon et al. 2007), or induce apoptosis or necrosis (Zagon and McLaughlin 2003), but rather upregulates the cyclin-dependent inhibitory kinase pathway, specifically p16 and p21 (Cheng et al. 2009b, 2007a,b). OGFr is localized on the outer nuclear envelope, and the OGF-OGFr complex undergoes nucleocytoplasmic transport using nuclear localization signals encoded in OGFr for guidance by karyopherin β/Ran through the nuclear pore (Zagon et al. 2005a; Cheng et al. 2009a). Blockade of OGF from OGFr by opioid antagonists or antisense strategies, as well as neutralization of OGF by antibodies, accelerates the pace of cell proliferation (Donahue et al. 2009; Zagon et al. 2005b).

A number of reports have linked the OGF-OGFr system to autoimmune diseases (Zagon et al. 2009b, 2010). In a study by Zagon et al. (2010), OGF repressed the incidence and magnitude of myelin oligodendrocyte-induced experimental autoimmune encephalomyelitis (EAE) in mice. Given the extensive connection between the immune system and autoimmune diseases, the present study was conducted to investigate the relationship of the OGF-OGFr axis and regulation of B lymphocyte proliferation *in vitro*.

Materials and methods

Mice

Four to 6-week-old C57BL/6 male and female mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in-house. All mice were housed in a controlled-temperature room (22–25 °C) with a 12–12 h light/dark cycle (lights on 07:00–19:00). Standard rodent diet (Harlan Teklad, Indianapolis, IN; catalog number 2018) and water were available *ad libitum*.

Isolation of lymphocytes

Lymphocytes were isolated from the spleens of adult mice that were euthanized by cervical dislocation. Spleens were removed, and mechanically dissociated with 60-mesh stainless steel screens (Sigma Aldrich, St. Louis, MO). Cells were collected and red blood cells lysed in a hypotonic saline solution (17 mM Tris, 0.14 mM NH_4Cl, pH 7.65) for 5 min at 37 °C. The resulting lymphocytes were counted by trypan blue dye exclusion and suspended in Iscove's modified Dulbecco's Media (IMDM) supplemented with 10% (v/v) fetal bovine serum (Gibco, Carlsbad, CA), 0.075% (w/v) sodium bicarbonate, 0.00035% (v/v) β-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate. The splenic-derived lymphocytes were either seeded onto slides for immunohistochemical studies or cultured in 24- or 96-well flat-bottom plates for proliferation studies. For all experiments, cells were incubated in a humidified atmosphere at 37 °C with 5% CO_2.

Immunohistochemistry

To determine whether B lymphocytes express OGF and/or OGFr, 5×10^5 splenic-derived lymphocytes were added into 24-well plates containing 500 μl of supplemented IMDM, and were either left as non-stimulated or were stimulated with 10 μg/ml of the B cell mitogen lipopolysaccharide (LPS) (Sigma Aldrich) for 72 h. Subsequently, cells were harvested, counted, and resuspended at 1×10^6 cells/ml in supplemented IMDM. Two hundred μl of the cell suspension was placed on glass slides and allowed to dry overnight. Preparations were fixed and permeabilized with 95% (v/v) ice-cold ethanol and 100% acetone, and processed for immunohistochemistry using antibodies to OGF, OGFr, and CD19 according to published procedures (Donahue et al. 2009). Polyclonal antibodies to OGF and OGFr were generated in our laboratory and have been fully characterized (Zagon and McLaughlin 1993), while rat anti-mouse CD19 was purchased from eBioscience (clone 1D3, San Diego, CA). Some cells were incubated with secondary antibodies only (rabbit anti-rat IgG, FITC, ab6730D, Abcam, Cambridge, MA; goat anti-rabbit IgG, TRITC, A11011, InVitrogen, Carlsbad, CA) and served as negative controls. All cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei. At least four slides (containing at least 500 cells/slide) per condition were examined.

Receptor binding assays

Receptor binding assays for OGFr were performed using freshly isolated lymphocytes and custom synthesized [^{3}H]-[Met^5]-enkephalin (Perkin Elmer-New England Nuclear; 52.7 Ci/mmol) according to previously published procedures (Donahue et al. 2009). Splenocytes from three animals were pooled for each assay; independent assays were performed at least three times.

Mitogen-induced lymphocyte proliferation

For lymphocyte proliferation assays, 5×10^5 splenic-derived lymphocytes were cultured in 500 μl supplemented IMDM in 24-well plates, and were either non-stimulated or stimulated with 10 μg/ml (unless otherwise specified) of LPS for 72 h; in some studies cells were stimulated with 5 μg/ml LPS. Immediately upon the addition of LPS, cells were treated daily with compounds; neither media nor compounds were replaced. All compounds were prepared in sterile water and dilutions represent final concentrations. An equivalent volume of sterile water was added to control wells. At designated times, cells were collected, stained with trypan blue, and counted using a hemacytometer. At least two aliquots per well and at least two wells/treatment/timepoint were evaluated.

Analysis of lymphocyte cell surface markers

Flow cytometric analysis of lymphocyte cell surface markers was determined as described previously (Nair and Bonneau 2006). Briefly, lymphocytes were either non-stimulated, or stimulated with LPS for 72 h. CD16/CD32 Fcγ receptors were blocked with an antibody obtained from 2.4G2 hybridoma cell culture supernatants (a gift from Dr. Bonneau) supplemented with 10% mouse serum (Sigma Aldrich). Cell surface expression of CD4, CD8, and B220 were detected using anti-CD4 FITC (clone GK1.5), anti-CD8a FITC (clone 53-6.7; eBioscience), and anti-CD45R FITC (clone RA3-6B2) antibodies, respectively; all antibodies were obtained from eBioscience. Following washes with FACS buffer (Hank's buffered saline solution supplemented with 1% (w/v) BSA), cells were resuspended in FACS buffer and immediately analyzed by flow cytometry.

Specificity of endogenous OGF

The specificity of endogenous OGF for lymphocyte growth was evaluated by treating LPS-stimulated lymphocytes with a rabbit polyclonal antibody to OGF (1:200; Co172) (Zagon and McLaughlin 1993); pre-immune rabbit serum (1:200; IgG) served as a control. Cells were treated with antibody, serum, or sterile water daily without replenishing media, and cell number was determined following 72 h of treatment, with at least two aliquots/well and at least two wells/treatment counted.

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