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# T lymphocyte proliferation is suppressed by the opioid growth factor ([Met$^5$]-enkephalin)–opioid growth factor receptor axis: Implication for the treatment of autoimmune diseases

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ABSTRACT

Opioid peptides function as immunomodulatory molecules. Reports have linked the opioid growth factor (OGF), [Met$^5$]-enkephalin, and its receptor OGFr to autoimmune diseases. OGF repressed the incidence and magnitude of myelin oligodendrocyte-induced experimental autoimmune encephalomyelitis in mice. Given the extensive connection between the immune system and autoimmune diseases, the present study was conducted to examine the relationship of the OGF–OGFr axis and T lymphocyte proliferation. Splenic-derived mouse lymphocytes were stimulated with phytohemagglutin (PHA). All non-stimulated and PHA-stimulated T lymphocytes had immunoreactivity for OGF-like enkephalin and OGFr. OGF markedly suppressed T lymphocyte number in a dose-dependent manner. However, PHA-stimulated T lymphocytes were not altered in cell number by a variety of natural and synthetic opioid-related compounds, some specific for μ, δ, and κ opioid receptors. Persistent blockade of opioid receptors with the general opioid antagonist naltrexone (NTX), as well as antibody neutralization of OGF-like peptides, had no effect on cell number. Non-stimulated T lymphocytes exhibited no change in cell number when subjected to OGF or NTX. Treatment of T lymphocytes with siRNAs for μ, δ, or κ opioid receptors did not affect cell number, and the addition of OGF to these siRNA-exposed cultures depressed the population of cells. T lymphocytes treated with OGFr siRNA also had a comparable number of cells to control cultures, but the addition of OGF did not alter cell number. DNA synthesis in PHA-stimulated T lymphocytes exposed to OGF was markedly decreased from PHA-stimulated cultures receiving vehicle, but the number of cells undergoing apoptosis or necrosis in these cultures was similar to control levels. T lymphocytes subjected to siRNA for p16 and/or p21 had a comparable number of cells compared to controls, and treatment with OGF did not depress cell number in preparations transfected with both p16 and p21 siRNA. These data reveal that the OGF–OGFr axis is present in T lymphocytes and is capable of suppressing cell proliferation. However, T lymphocytes are not dependent on the regulation of cell proliferation by this system. The results showing that the OGF–OGFr axis is an immunosuppressant, offers explanation for reports that autoimmune diseases can be modulated by this system.



## Introduction

Opiates and endogenous opioid peptides (referred to collectively as opioids) function as 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). The three classical opioid receptors, μ (MOR), δ (DOR), and κ (KOR), as well as nonclassical opioid receptors (e.g., nociceptin/ophanin FQ) have been found on immune cells in multiple studies using pharmacological and cellular/molecular approaches (Carr et al. 1996; McCarthy et al. 2001; Sharp et al. 1998; Sharp 2004; Chuang et al. 1994, 1995). Opioid peptide gene and protein expression for the three families of endogenous opioids: pro-opiomelanocortin, proenkephalin, and prodynorphin have been reported for cells of the immune system (Roth et al. 1989; Linner et al. 1995; Kamphuis et al. 1998; Lolait et al. 1986; Martin et al. 1987; Smith and Blalock 1981). The picture emerging about the role of the endogenous opioids often indicates complex and divergent effects of these peptides that may be dependent or independent on opioid receptors.

[Met$^5$]-enkephalin is an endogenous opioid peptide derived from preproenkephalin (Akil et al. 1984; Noda et al. 1982). This

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peptide was 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). This peptide has been termed the opioid growth factor (OGF) to signify its distinct distribution and biological role (Zagon et al. 2002). OGF action is mediated by the OGF receptor (OGFr). Although OGF–OGFr has the same pharmacological properties of opioid peptides that interact with classical opioid receptors (e.g., blockade by naloxone and stereospecificity), OGFr has nucleotide and protein sequences that are entirely different from that of classical opioid receptors (Zagon et al. 2002). OGF–OGFr interactions 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, 1998; Wilson et al. 2000; Blebea et al. 2000; McLaughlin et al. 2005). The OGF–OGFr axis upregulates the cyclin dependent inhibitory kinase pathway, specifically p16 and p21 (Cheng et al. 2007a,b, 2009a), and does not induce apoptosis or necrosis (Zagon and McLaughlin 2003), or differentiation (Zagon and McLaughlin 2005). OGFr is localized on the outer nuclear envelope, and the OGF–OGFr complex undergoes nucleocytoplasmic transport using nuclear localization signals encoded on OGFr for guidance by karyopherin β and Ran through the nuclear pore (Zagon et al. 2005a; Cheng et al. 2009b, 2010a). Blockade of OGF from OGFr by opioid antagonists, antisense strategies, or siRNA technology, as well as neutralization of OGF by antibodies, accelerates the pace of cell proliferation (Zagon et al. 2005b; Donahue et al. 2009).

A number of reports have linked the OGF–OGFr system to autoimmune diseases (Zagon et al. 2009a, 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. Splenocytes obtained from normal mice and stimulated by phytohemagglutin (PHA) were found to be depressed in cell number by OGF. Activation of the OGF–OGFr axis also is known to decrease tumor incidence, delay tumor growth, and extend survival in mice with xenografts of human cancer (McLaughlin et al. 2008; Zagon et al. 2008a). In view of the connection between the immune system and autoimmune diseases, as well as neoplasia, the present study was conducted to investigate fully the relationship of the OGF–OGFr axis and T lymphocyte proliferation.

## Materials and methods

### *Mice*

Four to six-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, 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 the 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*

For immunohistochemical studies, $5 \times 10^5$ splenic-derived lymphocytes were added in 500 μl supplemented IMDM in 24-well plates, and were either left as non-stimulated, or were stimulated with 10 μg/ml of PHA (Sigma–Aldrich) for 72 h. Subsequently, cells were harvested, counted, and resuspended at $1 \times 10^6$ 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 CD3 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 CD3 was purchased from eBioscience (clone 17A2, 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 4 slides (containing at least 500 cells/slide) per condition were examined.

### *Mitogen-induced lymphocyte proliferation*

For lymphocyte proliferation assays, $5 \times 10^5$ splenic-derived lymphocytes were cultured in 500 μl supplemented IMDM in 24-well plates, and were either non-stimulated or were stimulated with 5 or 10 μg/ml of PHA for 72 h. Immediately upon the addition of PHA, cells were treated daily with compounds; media and compounds were not 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 2 wells/treatment/time point were counted.

### *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 10 μg/ml PHA for 72 h. CD16/CD32 Fcγ receptors were blocked with an antibody obtained from 2.4G2 hybridoma cell culture supernatants supplemented with 10% mouse serum (Sigma–Aldrich). Cell surface-expression of CD4, CD8, and B220 were detected using anti-CD4 FITC antibody (clone GK1.5; eBioscience), anti-CD8a FITC antibody (clone 53-6.7; eBioscience), and anti-CD45R FITC antibody (clone RA3-6B2, eBioscience), respectively. 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 PHA (10 μg/ml)-stimulated lymphocytes with a rabbit polyclonal antibody to OGF (1:200; Co172) (Zagon and McLaughlin 1993); pre-immune rabbit serum (1:200; IgG)