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Short Communication

CD4$^+$ T cell-mediated neuroprotection is independent of T cell-derived BDNF in a mouse facial nerve axotomy model

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ABSTRACT

Background: The production of neurotrophic factors, such as BDNF, has generally been considered an important mechanism of immune-mediated neuroprotection. However, the ability of T cells to produce BDNF remains controversial.
Methods: In the present study, we examined mRNA and protein of BDNF using RT-PCR and western blot, respectively, in purified and reactivated CD4$^+$ T cells. In addition, to determine the role of BDNF derived from CD4$^+$ T cells, the BDNF gene was specifically deleted in T cells using the Cre-lox mouse model system.
Results: Our results indicate that while both mRNA expression and protein secretion of BDNF in reactivated T cells were detected at 24 h, only protein could be detected at 72 h after reactivation. The results suggest a transient up-regulation of BDNF mRNA in reactivated T cells. Furthermore, in contrast to our hypothesis that the BDNF expression is necessary for CD4$^+$ T cells to mediate neuroprotection, mice with CD4$^+$ T cells lacking BDNF expression demonstrated a similar level of facial motoneuron survival compared to their littermates that expressed BDNF, and both levels were comparable to wild-type. The results suggest that the deletion of BDNF did not impair CD4$^+$ T cell-mediated neuroprotection.
Conclusion: Collectively, while CD4$^+$ T cells are a potential source of BDNF after nerve injury, production of BDNF is not necessary for CD4$^+$ T cells to mediate their neuroprotective effects.

1. Introduction

Our laboratory has previously shown that compared to wild type (WT) mice, immunodeficient mice lacking T and B cells have a significant decrease in motoneuron survival, which can be rescued by adoptive transfer of CD4$^+$ T cells (Serpe et al., 1999, 2003). While multiple effector subsets of CD4$^+$ T cells developed after facial nerve axotomy (Xin et al., 2008), mice with an impaired Th2 response exhibit a decrease in motoneuron survival after axotomy (Deboy et al., 2006). More recently, we determined that IL-10, a Th2-secreted anti-inflammatory cytokine, plays a critical role in supporting facial motoneuron survival after nerve injury, but that CD4$^+$ T cells were not the primary source of IL-10 (Xin et al., 2011). Thus, the molecular mechanism responsible for CD4$^+$ T cell-mediated neuroprotection remains to be elucidated.

Brain-derived neurotrophic factor (BDNF) and its receptors are expressed in the thymus and may play an important role in T cell survival (Maroder et al., 1996; De Santi et al., 2009; Azoulay et al., 2008). It has also been demonstrated that mature resting CD4$^+$ T cells express a low level of BDNF (Kerschensteiner et al., 1999; Ziemssen et al., 2002). Previously, our laboratory determined that lymph node cells, isolated from mice 9 days post-axotomy and reactivated *in vitro* for 24 h, are capable of secreting BDNF, and we proposed that the release of BDNF may underlie the mechanism of immune-mediated neuroprotection by the CD4$^+$ T cells following nerve injury (Serpe et al., 2003, 2005). In the current study, using mice with T cells depleted of BDNF, we examined the ability of those cells to support facial motoneuron (FMN) survival after a facial nerve axotomy. Our results indicate that CD4$^+$ T cells are capable of producing BDNF, however, to our surprise, that production is not required for T cell-mediated neuroprotection of motoneurons from axotomy-induced cell death.

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2. Materials and methods

2.1. Animals and surgical procedures

Seven-week-old female C57Bl/6 wild-type and transgenic mice were obtained from Jackson Laboratory (Sacramento, CA, USA). Two transgenic groups of mice were used to create conditional BDNF gene knockout mice. One group, Lck-Cre, bears the Cre-recombinase gene driven by the distal promoter of the lymphocyte protein tyrosine kinase (Lck), which is a T cell receptor signaling component and only observed in T cells after T cell receptor α (Tcra) locus rearrangement. The second group possesses *loxP* sites on either side of exon 5 of the BDNF gene. Upon breeding these two groups, the litters contained two genotypes of mice, one $Cre^{+/-}$ genotype, expressing the Cre gene in T cells and leads to the deletion of the BDNF gene in T cells, and the other $Cre^{-/-}$ genotype, which does not express the Cre and the BDNF gene in T cells remains intact. These mice were bred and prepared by Jackson laboratory. All mice were housed and surgery was performed as previously published (Serpe et al., 2003). All surgical procedures were completed in accordance with National Institutes of Health guidelines on the care and use of laboratory animals for research purposes.

2.2. Preparation of $CD4^+$ T cells and reactivation

Right (draining) cervical lymph nodes were collected from axotomized mice ($n = 4$/group) at 9 day post operative, and then $CD4^+$ T cells were isolated via autoMACS using anti-CD4 magnetic beads as previously published (Xin et al., 2008). $CD4^+$ T cells were plated in two sets of culture cambers with or without anti-CD3 coating. The cells that received anti-CD3 stimulation were defined as reactivated cells, because these cells were first activated by axotomy *in vivo*. Cells and supernatants were harvested at two time points, 24 and 72 h. Cells were used for RNA extraction and RT-PCR (Invitrogen, Carlsbad, CA). The supernatants were subjected to Western blot analysis.

2.3. RNA preparation, RT-PCR, and electrophoresis

Complementary DNA was used in RT-PCR reactions in an iCycler (Applied Biosystems, Foster City, CA). Twenty-five microliter PCR reactions contained 1× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 1 μL cDNA, and 200 nM forward and reverse primers. RT-PCR cycle parameters included an initial 95 °C for 10 min, followed by 45 cycles of 95 °C 30 s, 54 °C 30 s and 65 °C 30 s. BDNF PCR primers were designed from published mouse sequences, forward: 5′-CCATAAGGACGCGGACTTG-3′; reverse: 5′-GACATGTTTGCGGCATCCA-3′. PCR products were separated on Criterion precast 10% nondenaturing polyacrylamide TBE gels (BioRad, Hercules, CA) for 90 min at 100 V. Gels were imaged on a STORM 860 Phosphoimager using Storm Scanner and ImageQuant programs. The PCR for detection of BDNF was performed using DNA samples from cervical lymph node cells with the method provided by Jackson Lab.

2.4. Western blot analysis

The protein concentration of each sample was determined by Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Samples were separated on an 12% Ready Gel Tris–HCl Gel (Bio-Rad Laboratories, Hercules, CA) at 150 V for 45 min, and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ) at 100 V for 60 min. The remainder of the Western blot analysis was accomplished by using a Protein Detector Lumi-GLO Reserve Western Blotting Kit (KPL, Gaithersburg, Maryland) following manufacture's manual. An anti-human BDNF polyclonal antibody (1:1000) (Promega, San Luis Obispo CA) was used as primary antibody, and HRP-labeled anti-chicken IgG secondary antibody (1:500) (KPL, Gaithersburg, Maryland). Positive identification of BDNF was determined by a 27 kDa band (High-Range Rainbow Molecular Weight Marker; Amersham Biosciences).

2.5. Surface and intracellular staining, and flow cytometric analysis

Single cell suspension of draining cervical lymph node cells were first incubated with phorbol myristate acetate (PMA, 50 ng/ml) and ionomycin (500 ng/ml) for 6 h in the presence of brefeldin A (10 μg/ml) during the final 2 h. The T cells were permeabilized with Saponin and doubly stained with anti-CD4-APC (BD Pharmingen, San Diego, CA) and chicken anti-human BDNF (Promega, San Luis Obispo, CA). The stained cells were subjected to multi-color FACS analysis.

2.6. Tissue sectioning and cell counts, statistical analysis

Coronal sections of the brainstem containing the facial nuclei were thaw-mounted onto SuperFrost Plus slides (Fisher) and facial motoneurons were counted under blind conditions as previously described (Serpe et al., 1999). Facial motoneuron survival was expressed as a percentage by comparing the number of cells on the right (injured) side to left (uninjured) side. The counting correction factor and section alignment procedures have been described in previous reports (Jones and LaVelle, 1985). Data are expressed as mean ± SEM. One-way ANOVA was performed to determine statistical differences among experimental groups at $p < 0.05$.

3. Results

3.1. Expression of BDNF mRNA and protein by $CD4^+$ T cells

$CD4^+$ T cells from axotomized mice were reactivated *in vitro* with anti-CD3 or non-reactivated, without anti-CD3, for 24 or 72 h. As shown in Fig. 1A, following anti-CD3 reactivation, BDNF mRNA expression in $CD4^+$ T cells was detected at 24 h, but not 72 h after anti-CD3 reactivation. Without reactivation, BDNF mRNA expression in $CD4^+$ T cells was undetectable. These results suggest that $CD4^+$ T cells express detectable levels of BDNF mRNA after being activated via injury and reactivated *in vitro*. In contrast, BDNF protein expression was detectable at 24 and 72 h in culture with and without anti-CD3 reactivation (Fig. 1B). However, the pattern of BDNF expression in the cell culture supernatant differs from the mRNA expression. First, BDNF protein was present in the culture supernatants regardless of whether or not the cells were activated with anti-CD3. Second, BDNF protein was secreted by $CD4^+$ T cells in cervical lymph node from both axotomized and uninjured mice, suggesting that facial axotomy is not a requisite for $CD4^+$ T cells to acquire BDNF-producing capability.

3.2. Facial motoneuron survival in the presence or absence of BDNF expression by T cells

As detailed in the methods, two groups of transgenic mice, Lck-Cre and Loxp-BDNF, were used to create offspring with a conditional knockout of the BDNF gene (Fig. 2A). To confirm the successful deletion of BDNF gene expression in the $Cre^{+/-}$ mice, lymph node cells from $Cre^{-/-}$ and $Cre^{+/-}$ mice underwent PCR detection of BDNF gene and anti-BDNF staining for protein expression. The deletion of BDNF gene was detected, as shown by bands with a larger size in $Cre^{+/-}$ mice (Fig. 2B). Of note, the intact BDNF