Institut Pasteur
Microbes and Infection 16 (2014) 702–706
www.elsevier.com/locate/micinf

Short communication

Nerve growth factor promotes killing of *Leishmania donovani* by macrophages through the induction of hydrogen peroxide

Rieko Chiba [a], Yosuke Amagai [a], Akane Tanaka [b], Ken Katakura [c], Hiroshi Matsuda [a,*]

[a] *Laboratories of Veterinary Molecular Pathology and Therapeutics, Division of Animal Life Science, Tokyo University of Agriculture and Technology, 3-5-8, Saiwai-cho, Fuchu, Tokyo 183-8509, Japan*

[b] *Laboratories of Comparative Animal Medicine, Division of Animal Life Science, Tokyo University of Agriculture and Technology, 3-5-8, Saiwai-cho, Fuchu, Tokyo 183-8509, Japan*

[c] *Laboratory of Parasitology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan*

Received 4 April 2014; accepted 2 June 2014
Available online 14 June 2014

Abstract

Visceral leishmaniasis is protozoonosis that occurs worldwide and still requires effective therapies with less toxicity. In this study, we examined the antileishmanial effect of nerve growth factor (NGF) using a murine infection model. NGF blocked the infection of macrophages by *Leishmania donovani*, which was completely cancelled by a hydrogen peroxide inhibitor. In vivo, not only did NGF show antileishmanial effects, but combination therapy of NGF and sodium stibogluconate synergistically exhibited the activity more potently than each monotherapy. These results indicate that NGF exerts antileishmanial effect by stimulating hydrogen peroxide production in macrophages and can be a novel therapy for leishmaniasis.

Keywords: Peroxide; *Leishmania*; Macrophages; Nerve growth factor

1. Introduction

Leishmaniasis is intracellular protozoan infections transmitted by the bite of sandfly [1]. The clinical outcomes of the disease are diverse; cutaneous, visceral and mucocutaneous forms have been reported [1]. Among these, visceral leishmaniasis, which is caused by *Leishmania donovani* (*L. donovani*), *Leishmania chagasi*, or *Leishmania infantum*, exhibits the most severe symptoms represented by the pancytopenia and enlarged liver/spleen [1,2]. Though pentavalent antimonial agents and amphotericin B are effective for leishmaniasis, there are concerns about adverse effects such as cardiotoxicity, elevations of liver and pancreatic enzymes [3,4]. Because the parasites initially infect with the phagocytes in the reticuloendothelial system through the dissemination of parasites or infected macrophages of the initial infection site [2,5,6], immunomodulating agents that modify macrophage responses can be useful for suppressing exacerbation of the disease. For instance, interferon (IFN)-γ activates production of nitric oxide (NO) within macrophages, resulting in the death of parasites [7].

We previously reported that nerve growth factor (NGF) activated macrophages and eliminated *L. donovani* within the cells in vitro [8]. NGF is a neurotrophic polypeptide required for survival and maintenance of both the peripheral and central neurons [9]. It is released from various kinds of cells including keratinocytes, fibroblasts, vascular endothelial cells, T cells and mast cells [10–12] and serum concentrations of NGF are around 200 pg/mL in healthy adults [13]. Because NGF does not directly affect the survival of protozoa, it is assumed that

* Corresponding author. Tel.: +81 42 367 5784; fax: +81 42 367 5916.
E-mail address: hiro@cc.tuat.ac.jp (H. Matsuda).

http://dx.doi.org/10.1016/j.micinf.2014.06.001
1286-4579/

NGF modulated the response in macrophages. However, the mechanism of the antileishmanial effect in detail as well as therapeutic effect of NGF in vivo remained unknown. Therefore, in this study, we investigated antileishmanial effect of NGF both in vitro and in vivo and revealed the mechanism of its curative effect.

2. Materials and methods

2.1. Cytokine

2.5S NGF purified from murine submaxillary glands was kindly provided by Drs. A. M. Stanisz and J. Bienenstock (McMaster University, Hamilton, Ontario, Canada) [14].

2.2. Cell culture

J774A.1 murine macrophage cell line was cultured in RPMI1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% foetal bovine serum (Filtron, Brooklyn, Australia) and antibiotics.

2.3. Analysis of antileishmanial ability of NGF

2.3.1. In vitro model

Peritoneal macrophages were isolated 4 days after peritoneal injection with sodium caseinate (Wako, Tokyo, Japan) in 6–10 weeks old female BALB/c mice (CLEA Japan Inc., Tokyo, Japan). A total number of 2×10^5 cells were suspended in RPMI1640 containing 10% FBS, and were cultured in 8-chamber tissue culture slides for 2 h to allow cells to adhere. Then adherent macrophages were infected with *L. donovani* promastigotes (2S-25M-C2 line) [15] at a host: parasite ratio of 1:10 for 3 h. After washing, macrophages were incubated with various doses of NGF or IFN-γ for 72 h, and antileishmanial effects of NGF or IFN-γ were assessed by counting the number of parasites in at least 500 macrophages and were expressed as the percentage of infected cells. In some experiments, either 10 U/mL of glutathione peroxidase (GPX, Sigma Chemical, St Louis, MO) or 500 ng/mL of L-N^G-mono-methylarginine (L-NMMA, Sigma Chemical) was added simultaneously to the incubation of the macrophage.

2.3.2. In vivo model

Female BALB/c mice were injected intravenously with 1×10^8 *L. donovani* promastigotes on Day 0, then NGF and sodium stibogluconate (Glaxo SmithKline, Uxbridge, Middlesex, UK) were administered on Day 7, 8, and 9 by intraperitoneal and intramuscular injection, respectively. On Day 14, mice were euthanized, and the liver was removed and weighed; then impression smears were made on a transverse cut surface of the right medial lobe of the liver. Smears were fixed with methanol and stained with Giemsa solution. Then the number of amastigotes per 500–1000 liver cell nuclei was counted and multiplied by liver-weight to express the parasite load and defined as Leishman-Donovan units (LDU) [16].

2.4. Measurement of hydrogen peroxide (H_2O_2) and NO levels in culture media of macrophages

Peritoneal macrophages (1×10^6 cells) were cultured at 37 °C for 2 h, incubated with NGF or IFN-γ for 48 h, and the supernatants were collected. The concentrations of H_2O_2 and NO were determined by using horseradish peroxidase method [17] and Griess method, respectively. In this method, NO concentration was determined as nitric dioxide (NO_2), the oxidized form of NO.

2.5. Statistical analysis

Statistical analysis was conducted by ANOVA with Dunnett's or Tukey's multiple comparison of means test using statistical add-in software for EXCEL (Esumi Corp., Tokyo, Japan). The level of significance was set at $p < 0.05$.

3. Results

First, therapeutic potential of NGF on leishmanial infection in murine peritoneal macrophages was evaluated. After the exposure of *L. donovani* to macrophages, cells were incubated with either NGF or IFN-γ. While approximately 70% cells were infected with the parasites in medium alone, the proportion of infected cells was significantly decreased in a dose-dependent manner, reaching at 21.2% by 500 ng/mL NGF treatment (Fig. 1A). The effect was as potent as the one of the IFN-γ treatment (Fig. 1A and B).

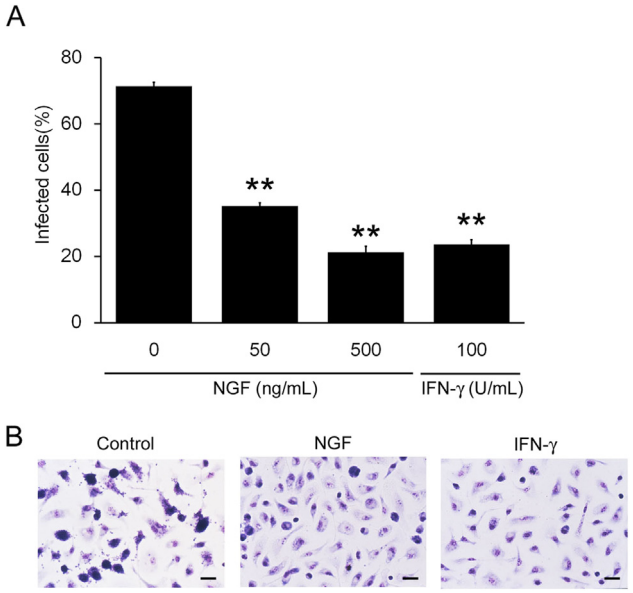

Fig. 1. Antileishmanial activity of NGF on infection of *L. donovani*. A. and B. Peritoneal macrophages of female BALB/c mice were infected with 2×10^6 *L. donovani* promastigotes, and then incubated in the presence or absence of either NGF or IFN-γ for 72 h. A. Data represent means ± SD of the percentage of infected macrophages in five randomly selected areas under a light microscope at a magnification of ×400. $^{**}p < 0.01$ compared to control. B. Representative data of infected macrophages treated with NGF or IFN-γ. Cells were treated with either 500 ng/mL NGF or 100 U/mL IFN-γ for 72 h, then fixed and stained. Original magnification was ×400. Bar: 30 μm.