Neurotrophin Expression in Chagasic Megacolon

Michelle Aparecida Ribeiro de Freitas1, Nathália Segatto2, Pedro Fellipe Remolli1, Enio Chaves de Oliveira1, Samir Jabari3, Axel Breherm4, Alexandre Barcelos Morais da Silveira2*

1Parasitology Sector, Universidade Federal de Uberlândia, Brazil
2Neurosciences Laboratory, Universidade Federal de Uberlândia, Brazil
3Department of Surgery, Universidade Federal de Goiás, Brazil
4Institute of Anatomy I, University of Erlangen–Nuremberg, Germany

ABSTRACT

Patients with chagasic megacolon exhibit lesions of the enteric nervous system (ENS), associated with the inflammatory process and, some substances, like neurotrophins, may restrict neuronal destruction levels. The objective of this study was to characterize the neurotrophins expression in tissues from chagasic patients and verify its involvement in megacolon development. We used colon samples from chagasic patients with and without megacolon and non-infected individuals that, after preparation, were submitted to confocal fluorescence immunohistochemistry. To identify the sources and expression level of neurotrophins Nerve growth factor (NGF), Glial cell-derived neurotrophic factor (GDNF), and Neurotrophin-3 (NT-3), we performed a co-localization with a neuronal marker (Calretinin) and glial cell marker (S-100). Our results pointed that enteroglial cells are the main sources of neurotrophins in all analyzed groups. Besides, chagasic patients without megacolon presented high expression of all investigated neurotrophins when compared with chagasic patients with megacolon and with non-infected individuals. This data suggest that neurotrophins might perform a protective function in ENS due an inflammatory process and prevent the megacolon installation. By the other side, chagasic patients that do not express an adequate level of neurotrophins may evolve to megacolon form. We believe that drugs administration qualified to elevate neurotrophins levels in the intestine could prevent the megacolon installation and maintain the normal function of the gastrointestinal tract.

ABBRVIATIONS

NGF: Nerve Growth Factor; GDNF: Glial Cell-Derived Neurotrophic Factor; NT-3: Neurotrophin-3; ENS: Enteric Nervous System

INTRODUCTION

Chagas’ disease represents a substantial health and socioeconomic problem in several countries. In particular, affects 0-10 million people in the Americas, with an additional 40 million people at risk [1]. Chagas’ disease exhibits two clinical phases: acute and chronic. The acute phase occurs after infection, beginning when the parasite enters the mammalian host. This phase may appear asymptomatic or accompanied by nonspecific symptoms such as fever, asthma, and headache [2]. The chronic phase, mainly, may last for the patient’s entire lifetime. It begins with the decline of parasitemia and absence of symptoms. Even after the nonspecific symptoms, some organs may still be affected, such as the gastrointestinal tract, taking the form of two main syndromes: megasophasagus that leads to dysphagia and regurgitation, and megacolon, conducting to severe constipation and fecal retention [3]. Both megasophasagus and megacolon show strong cellular and humoral immune responses. The mechanisms underlying the transition from asymptomatic to symptomatic are still unclear, but numerous factors must be involved, such as differences in parasite strain, parasite load, infection time, host genetic background and immune response [4-6].

Recent reports about chagasic megacolon suggest that disturbance of immune system and enteric nervous system (ENS) has also been associated with the development of gastrointestinal forms [7-9]. Some substances act in both systems and the most important class are the neurotrophic factors or neurotrophins. Neurotrophins are important for neuronal survival and development. It is accepted that these substances may represent a link between the nervous and immune systems and some studies demonstrated that an unsteadiness of neurotrophins may arise during inflammatory processes [10]. Neurotrophins include nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF) and neurotrophin-3 (NT-3). NGF concentration in human intestine is associated with increased inflammatory process in several diseases. GDNF act stimulating the outgrowth of neuronal projections, neuronal differentiation and are able to protect neurons from apoptosis under various conditions, while
NT3 mediates growth of human intestinal mast cell, which is known to participate in the regulation of gastrointestinal motility [11-13].

It has been suggested that these neurotrophins may play an important role in the pathophysiology of Chagas’ disease, as was demonstrated in other gastrointestinal diseases like ulcerative colitis and inflammatory bowel disease [14-17]. However, neurotrophins involvement in the development of chagasic megacolon is not determinate. To elucidate this, the aim of this study was to analyze and compare the neurotrophins expression and its’ relation with neuronal and enteroglial cells in colon samples from chagasic patients with and without chagasic megacolon and non-infected individuals.

MATERIALS AND METHODS

Patients and samples

Colon tissue samples were collected from chagasic patients with megacolon (n=12), chagasic patients without megacolon (n=12) and non-infected individuals (n=12). All chagasic patients have been followed for many years due to Chagas disease infection and were enrolled in this study because they developed only the megacolon clinical form. Patients were not matched in terms of age, gender or ethnic origin. Reasons for tissue resection were colon complications caused by Chagas’ disease whilst non-infected individuals had adenocarcinoma or diverticular disease as the main reasons. All surgical specimens were collected from the same topographic region of the colon (rectum and sigmoid) and patients were submitted to low anterior resection or Duhamel surgical procedure. The use of human tissues for these experiments was approved by the Ethics Committee of the University of Erlangen-Nuremberg as well as the Human Ethics Committee of the Federal University of Uberlândia (Brazil).

Samples were collected from dilated segment and they had, at least, 4 cm of length and the tissue circumference was variable according to the amount of dilatation. The samples were rinsed with phosphate buffered saline (pH 7.2-7.4) and fixed. During fixation, the gut samples were distended with fixative solution with phosphate buffered saline (pH 7.2-7.4) in phosphate-buffered saline - pH 7.2-7.4) using a syringe. Then, the inflated segment was opened, separated into small segments were placed in the fixative solution for 2-4 h. After this time, the inflated segment was opened, separated into small pieces and stored in 0.05 % thimerosal in 0.1 M phosphate buffered saline (pH 7.2-7.4) at 4°C. Alternately, short segments were pinned out in a Sylgardlined Petridish and transferred to 4% formalin in 0.1 M phosphate buffer (pH 7.4) at room temperature for 2-3 h. After several washes in 0.05 M TRIS-buffered saline (TBS; pH 7.4), three longitudinal muscle/myenteric plexus whole mounts (2 cm length, 1 cm width) per segment (derived from each patient) were prepared. The following day, the small segments of tissue were transferred to a mixture of PBS-sucrose-azide and OCT (Optimal Cutting Temperature) compound (Tissue Tek, Elkhart, IN, USA) at a ratio of 1:1 for a further 24 hours before being embedded in 100% OCT. Sections 12 μm thick were cut and mounted on microscope slides and dried for 1 hour at room temperature.

Immunohistochemical investigation

Triple-staining immunohistochemistry was conducted combining the neuronal marker peripherin and S-100 (pan-glial marker) antibodies with neurotrophin. Details of primary antibodies to their targets has been proven by several previous works (18-22). Sections were first incubated in 10% normal donkey serum (NDS) plus 1% v/v Triton X-100 for 1h. Incubation with primary antibodies was carried out for 24 hours at 4°C with diluted antiserum containing 10% NDS. Double or Triple-labeling was achieved using a combination of Peripherin and S-100 with NGF, GDNF and NT3 markers. Following incubation in primary antisera, preparations were rinsed in PBS (3 × 10 min) and then incubated for 1 hour at room temperature with secondary antibodies (Donkey anti-goat, donkey anti-mouse and donkey anti-rabbit, dilution 1:1000, Molecular Probes, Germany). Further 3 × 10-min washes in PBS were done before the tissue was mounted in fluorescence mounting medium.

The analysis was performed considering the specifically labeled area of, at least, 10 neuronal ganglia per patient in both the submucosal and the myenteric plexuses. Prior to incubation, they were viewed by using a confocal laser scanning microscope (Nikon Eclipse E1000-M, Tokyo, Japan) equipped with a confocal system (Nikon Digital Eclipse C1) with three channels (laser configuration: 488 nm argon laser, 543 nm helium-neon laser [both from Melles Griot, Carlsbad, Calif, USA], 638 nm diode laser [Coherent, Santa Clara, Calif, USA]). A 20× dry objective lens (numerical aperture: 0.75) was used with the zoom factor set to 2.0 in all scanning sessions. Pinhole and gain were adjusted equally in all negative control sessions and values were noted. The images were created using three different excitation wavelengths and the figures plates were prepared using the EZ-C1 FreeViewer program (Gold Version 3.30 build 647) from Nikon Corporation.

Anti-peripherin, a very used intestinal pan-neuronal marker, and anti-S-100, that shows strong cross-reactivity against human enteroglial cells, were used to determine the co-localization between neurotrophins and neuronal or enteroglial components. Sections through ganglia were selected randomly in a meandering fashion until a total of 3 mm² were analyzed in each ganglionated plexus. Single optical section images on the same focus plane were created in the ganglia by applying 2 different excitation wavelengths (488 nm argon laser, 543 nm helium-neon laser) Pictures were prepared using Confocal Assistant 4.02 and CorelDraw 13. For area analysis of reactive structures, the pictures were analyzed individually and merged, and the positive area was counted in square micrometer.

Statistics

Statistical analyses were conducted based on the non-parametric ANOVA-ONE WAY test, with the goal of detecting differences between the groups of patients. The significance level was p < 0.05 and all analyses were performed using the GraphPad Prim 3.0 software (San Diego, CA). The distribution of frequencies of all the variables and the measures of central tendency were calculated using various parameters: average, mean, percentiles, standard deviation. The associations between the dependent and independent variables were tested through...
Table 1: Primary and secondary antibodies.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Source</th>
<th>Company</th>
<th>Code</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti human Peripherin</td>
<td>Goat</td>
<td>Santa Cruz Biotechnology</td>
<td>SC-7604</td>
<td>ALEXA Fluor 488 Donkey anti-goat</td>
</tr>
<tr>
<td>Anti human S-100</td>
<td>Mouse</td>
<td>DAKO Ink</td>
<td>Z-0311</td>
<td>ALEXA Fluor 488 Donkey anti-mouse</td>
</tr>
<tr>
<td>Anti human NGF</td>
<td>Mouse</td>
<td>Santa Cruz Biotechnology</td>
<td>SC-549</td>
<td>ALEXA Fluor 555 Donkey anti-mouse</td>
</tr>
<tr>
<td>Anti human GDNF</td>
<td>Mouse</td>
<td>Santa Cruz Biotechnology</td>
<td>SC-13147</td>
<td>ALEXA Fluor 555 Donkey anti-mouse</td>
</tr>
<tr>
<td>Anti human NT3</td>
<td>Mouse</td>
<td>Santa Cruz Biotechnology</td>
<td>SC-33907</td>
<td>ALEXA Fluor 555 Donkey anti-mouse</td>
</tr>
</tbody>
</table>

bivariate and multivariate regression techniques (simple linear regression, multiple or logistical regression, according to the characteristics of the variable).

RESULTS AND DISCUSSION

Peripherin and S-100 expression

The quantitative data of peripherin and S-100 expression demonstrated that compared with non-infected individuals and chagasic patients without megacolon, chagasic patients with megacolon presented an intense denervation process that was revealed by peripherin immunoreactive area analysis. Besides, it was observed that while chagasic patients with megacolon showed enteroglial cells loss, chagasic patients without megacolon demonstrated increased expression of these cells. This data is according to with previous studies that demonstrated the denervation process in chagasic patients with megacolon [6,7,23].

Peripherin/ S-100/ neurotrophins immunoreactions

The co-localization of peripherin, S-100 and neurotrophins antibodies demonstrated that all analyzed neurotrophins (GDNF, NGF and NT3) were concentrated mainly in the neuronal plexuses in relation with the muscle layers in chagasic patients and non-infected individuals. However, when we compared both neuronal plexuses (submucosal and myenteric) there was no difference in proportional concentration between these two substances. The neurotrophins expression analysis showed that GDNF are NGF expressed mainly by neurons and, enteroglial cells present all neurotrophins in lower levels (Figure 1,2).

Figure 1 Relation between peripherin (neuronal marker) and neurotrophins expression. GDNF is expressed mainly outside of neurons (A) while both NGF (B) and NT3 (C) are expressed by neurons. These observations can be checked in the merged pictures.
Figure 2 Relation between S-100 (enteroglial marker) and neurotrophins expression. GDNF is highly expressed inside enteroglial cells (A). NGF (B) and NT3 (C) are expressed both inside and outside of glial components. These observations can be checked in the merged pictures.

Quantitative data of neurotrophins expression was performed by a relative proportion between neurotrophins (GDNF, NGF and NT3 area) and neurons (peripherin area). Our results indicated that chagasic patients without megacolon have an increased expression statistically relevant of all studied neurotrophins (GDNF, NGF and NT3) compared with non-infected individuals and with chagasic patients with megacolon (Table 2).

**DISCUSSION**

A central finding in the present study was the characterization of the neurotrophins in samples from chagasic patients with megacolon and non-infected individuals. This is the first study to evaluate the relation between neuronal destruction, enteroglial loss, and distribution of neurotrophins in patients with chagasic megacolon. Our previous data indicated that chagasic patients with megacolon present high level of inflammation, and this process leads to denervation and destruction of the glial component in the intestine [7,24]. Besides, we demonstrated that colon from chagasic patients increased the levels of GAP-43 during neuronal destruction process [25]. We believe that the high level of GAP-43 indicates an attempt to recover the damaged components or even avoid a bigger loss in the enteric nervous system cells.

In the last years, a study using the culture of enteroglial cells determined that under inflammatory conditions, the glial cells increase the production of neurotrophins [14]. This data corroborates with our results because the same increased level of

<table>
<thead>
<tr>
<th>Groups / neurotrophins</th>
<th>Peripherin</th>
<th>S-100</th>
<th>GDNF</th>
<th>NGF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Submucosal-plexus</td>
<td>Mienteric-plexus</td>
<td>Submucosal-plexus</td>
<td>Mienteric-plexus</td>
</tr>
<tr>
<td>Non-infected individuals</td>
<td>310 ± 29</td>
<td>344 ± 36</td>
<td>287 ± 22</td>
<td>301 ± 40</td>
</tr>
<tr>
<td>Chagasic patients without megacolon</td>
<td>286 ± 21</td>
<td>308 ± 33</td>
<td>415 ± 41</td>
<td>466 ± 47</td>
</tr>
<tr>
<td>Chagasic patients with megacolon</td>
<td>197a ± 21</td>
<td>144a ± 15</td>
<td>188a ± 17</td>
<td>214a ± 23</td>
</tr>
</tbody>
</table>

(a) Statistically significant difference between this group and non-infected individual
(b) Statistically significant difference between this group and chagasic patients without megacolon; (p < 0.05)
neurotrophins was found in enteroglial cells of chagasic patients without megacolon. Our results confirmed that, as much as enteroglial cells, neurons are important sources of neurotrophins. It was observed that the neurotrophins production is concentrated in the neuronal plexuses (submucosal and myenteric) and both neurons and enteroglial cells participate in this process.

It’s reasonable to suggest that the neuronal recovery should be made by neurotrophic factors, but it was necessary to identify them, their sources and where they should act. This study allowed us to infer how the enteric nervous system behaves under megacolon development, and only in Chagas disease we can observe a denervation process in the intestine caused by a parasite infection. Today, around 11 million of people are infected by Trypanosoma cruzi and they are able to develop the chagasic megacolon anytime [26]. Besides, understanding Chagas disease will allow us to comprehend other intestinal diseases and maybe point new and more effective targets to treatment.

In the last decades, it was demonstrated that immune system has an important role in the chagasic megacolon development. It was previously demonstrated that the inflammatory process presented in chagasic megacolon is constituted mainly by lymphocytes, macrophages, eosinophils and mast cells [4,23,27,28]. It was showed that the higher inflammatory process, the higher the neuronal loss and the megacolon severity. Most of these immune cells are sources of neurotrophins, and because of this, we may suggest that although they act promoting an inflammatory process to destroy the parasite in the tissue, they also secret neurotrophins as an attempt to save as many neurons as possible [29]. We can observe in Figure (1,2) some spots of neurotrophins around neuronal ganglia that do not match with neurons or enteroglial cells. These spots are probably immune cells producing neurotrophins. However, just small concentrations of neurotrophins are out of neuronal ganglia, what suggest that immune cells alone are not enough to produce a satisfactory amount of neurotrophins. We conclude that although the enteric nervous system acts as the main source of neurotrophins, it needs the immune system cooperation to fulfill the role of repairing the neural loss.

We believe that to know the expression pattern of neurotrophins and their receptors in ENS of patients with chagasic megacolon can identify key components that provide protection to neurons as well as the components that do not actively participate in its protection. As an example, we suggest the activation of TrkA and TrkB receptors by activating substances or use neurotrophins synergist drug, which could actually increase the levels of regeneration ENS. From these data, we are certain that we will be able to suggest new treatment modalities that address both patient recovery and prevention of worsening of his states.

CONCLUSION

Our results demonstrated that a relationship exists between the expression of neurotrophins on the gastrointestinal tract and the intensity of the inflammatory process in Chagas’ disease. Although it was established that Chagas disease patients with high expression of neurotrophins remain asymptomatic than those who do not have this adaptation. We believe that future studies will guide us for the immunomodulation of the gastrointestinal tract based on neurotrophin expression, which will allow us to prevent and treat a variety of inflammatory bowel disease.

ACKNOWLEDGEMENTS

This work was supported by funds from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and Deutsche Forschungsgemeinschaft.

REFERENCES


