

***Trypanosoma cruzi* infection and/or administration of the nonsteroidal anti-inflammatory nimesulide increase the number of colonic crypts overexpressing metallothioneins in rat colon carcinogenesis**

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Abstract

Trypanosoma cruzi infection and nonsteroidal anti-inflammatory drugs inhibit colorectal carcinogenesis by mechanisms not completely known and metallothionein proteins (MTs) may be involved in this process. Sixty-six male Wistar rats weighing 90 to 120 g were randomly divided into seven groups (GI to GVII). GI, GII and GIII animals were subcutaneously infected with 200,000 trypomastigote forms of the Y strain of *T. cruzi*. After 8 weeks, GI, GII, GIV, and GVI were injected with one weekly subcutaneous dose of 12 mg/kg dimethylhydrazine for 4 weeks. In sequence, GI, GIV and GV were treated with nimesulide (10 mg/kg per dose, five times per week for 8 weeks). Groups I, III, IV, and VI had 12 animals, and each of the other groups had 6 animals. All the animals were euthanized 8 weeks after the last dimethylhydrazine injection. The colons were fixed and processed for MT immunohistochemistry. The index of MT-overexpressing colonic crypts (MTEC) was estimated as the percentage of MT-stained crypts in relation to the total number of crypts scored. Five hundred crypts per animal were scored. Data were analyzed by the Kruskal-Wallis test followed by the Dunn test. There was an increase in MTEC index in the groups either infected with *T. cruzi* or treated with nimesulide or both infected and treated when compared to control (401, 809, and 1011%, respectively). We suggest that the increased formation of MTEC may be related to the protection against carcinogenesis provided both by *T. cruzi* infection and nimesulide.

Key words

- *Trypanosoma cruzi*
- Nimesulide
- Dimethylhydrazine
- Metallothioneins
- Colon carcinogenesis

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Introduction

It has been known for decades that *Trypanosoma cruzi* infection has an anticancer effect (1) and we have provided evidence showing that chronic infection with *T. cruzi* reduces the incidence of 1,2-dimethylhydrazine-induced colorectal tumors (2). Available clinical data strongly suggest the existence of protection against colon cancer in patients with megacolon due to Chagas' disease, which is caused by *T. cruzi* (3). However, the mechanisms for this protection against cancer development are not known.

The observations that nonsteroidal anti-inflammatory drugs (NSAIDs) may inhibit colorectal carcinogenesis have also produced great excitement, despite the fact that comparative data relating their chemopreventive effectiveness to relevant mechanisms of action are not available (4). It has been reported that nimesulide, a cyclooxygenase-2 selective inhibitor, showed a remarkable suppressive effect on rodent carcinogenesis but the mechanisms were not identified (5).

Interestingly, it has been known that both infections in general and NSAIDs may cause a marked increase of metallothionein (MT) expression (6-8). The MTs are low-molecu-

lar weight proteins that are induced by exposure to heavy metals and which perform many functions such as detoxification and clearance of free radicals from tissues and cells (9). Recently, it has been shown that the MTs played some protective roles during DNA damage, presumably by acting as antioxidants (10). The carcinogen dimethylhydrazine (DMH) induces the appearance of MT-overexpressing colonic crypts (MTEC) in rodents. They are randomly distributed in the epithelium and presumably arise from somatic mutation of crypt stem cells followed by colonization of the clonal unit by the mutated progeny (9,11). It has been postulated that MT overexpression may represent an important early step in the development of colonic cancer (12).

Since MTs are antioxidants and their expression has been related both to colon carcinogenesis and to infections and inflammation, the present study was designed to determine if the relative number of MT-overexpressing colonic crypts is altered during colon carcinogenesis in rats by infection with *T. cruzi* and/or treatment with nimesulide.

Material and Methods

Sixty-six male Wistar rats bred at the Medical School of Ribeirão Preto, weighing 90 to 120 g at the beginning of the experiment were used. Water and a standard pellet diet (Labina, Ralston Purina do Brasil, São Paulo, SP, Brazil) were provided *ad libitum*. The animals were maintained in cages (5 per cage) on a 12-h light/dark cycle. The animal welfare considerations were in strict accordance with international bioethical guidelines.

The experimental design is summarized in Table 1. The animals were randomly divided into seven groups, with 12 animals each in groups I, III, IV, and VI at the beginning of the experiment, and 6 animals in each of the other groups.

The animals were infected with 200,000

Table 1. Experimental design.

| | Infection | 8 weeks | 4 weeks | 8 weeks |
|--------------------|-----------|---------|---------|---------|
| Group I (N = 11) | ▲ | - | DMH | NMS |
| Group II (N = 6) | ▲ | - | DMH | - |
| Group III (N = 12) | ▲ | - | - | - |
| Group IV (N = 12) | - | - | DMH | NMS |
| Group V (N = 6) | - | - | - | NMS |
| Group VI (N = 12) | - | - | DMH | - |
| Group VII (N = 6) | - | - | - | - |

After 8 weeks of infection, rats were treated with DMH for 4 weeks, which was discontinued at 12 weeks, when 8 weeks of NMS was started. Twenty weeks after the initial infection all rats were killed. Triangle = *Trypanosoma cruzi* infection (200,000 forms of Y strain of *T. cruzi*, sc); DMH = dimethylhydrazine treatment with one weekly subcutaneous dose of 12 mg/kg DMH; NMS = treatment with nimesulide (10 mg/kg per dose, five times per week); N = number of animals at the end of the experimental period.

trypomastigote forms of the Y strain of *T. cruzi* by the subcutaneous route. The infection was monitored by checking the parasitemia 5 days after infection by light microscopic examination of a blood smear (2). The animals were treated with one weekly subcutaneous dose of 12 mg/kg DMH for 4 weeks. Nimesulide was administered at 10 mg/kg per dose, five times per week for 8 weeks. The animals were killed by cervical dislocation. The colons were fixed and processed histologically for MT immunohistochemistry as previously described (9). Briefly, colorecta were removed, coiled into Swiss-rolls, fixed in neutral buffered formalin, and paraffin-embedded. Multiple 5- μ m sections were cut at 40- μ m intervals to provide a sample of at least 45,000 crypts per mouse. The sections were immunoperoxidase-stained for MT using the ascites-derived mouse monoclonal antibody E9 (Dako Ltd., Copenhagen, Denmark), which reacts with *MT-I* and *MT-II*,18 applied at 0.1 μ g/mL (IgG, K protein) overnight at 4°C. Normal adult rat endometrium (in which MT is consistently expressed in the glandular epithelium) was used as a positive control, while the primary antibody was excluded for negative controls.

The MTEC index was reported as the percentage of MT-stained crypts in relation to the total number of crypts scored. Five hundred crypts per animal were scored by two pathologists who were blind to group identification. Data were analyzed statistically by the Kruskal-Wallis test followed by the Dunn test. Differences were considered to be significant when $P \leq 0.05$.

Results and Discussion

MT-positive crypts were found only in the animals treated with the DMH. In these animals, both *T. cruzi* infection and nimesulide ingestion were associated with an increase in the number of MT-overexpressing colonic epithelial cells when compared to

the control group. Furthermore, there was an even greater increase in MT-positive crypts when *T. cruzi* infection was associated with nimesulide, as shown in Figures 1 and 2.

It has been observed that colorectal carcinogenesis is characterized by a significant decrease in MT expression (13). Conversely, in some conditions MT expression is consid-

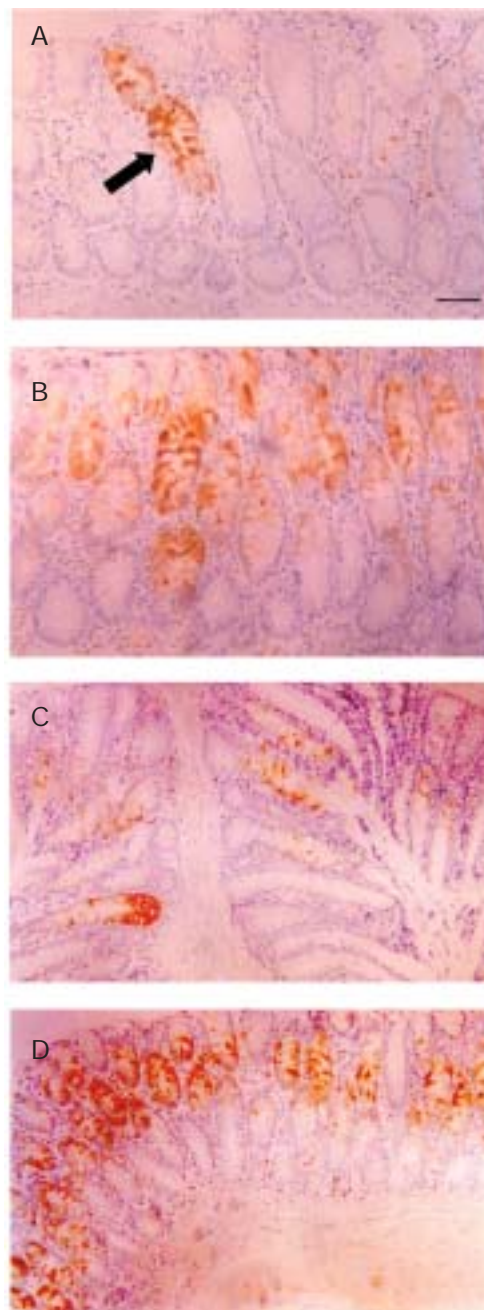


Figure 1. Immunohistochemistry of metallothioneins (MT) in microscopically normal colorectal mucosa of rats treated with dimethylhydrazine (magnification, 200X). A, Colon section of a control animal with only one colonic crypt overexpressing MT (arrow) and surrounding crypts with no MT expression. B, Rat infected with *Trypanosoma cruzi*, showing MT overexpression in many crypts. C, Rat treated with nimesulide, showing MT overexpression in many crypts. D, Rat infected with *T. cruzi* and treated with nimesulide. Most of the crypts are positive for MTs (Bar = 100 μ m).

ered to be protective against cancer development or to indicate a better prognosis (14). Both the *T. cruzi* infection and nimesulide have been reported to protect against colonic carcinogenesis (2,4).

Here we demonstrate that *T. cruzi* infection and nimesulide administration were associated with an increase in colonic MT expression after DMH injection in rats. Furthermore, there was an even greater increase in relative number of MT-expressing crypts when *T. cruzi* infection was associated with nimesulide, suggesting that there may be a cumulative effect of *T. cruzi* infection and NSAID. The oncological relevance of MTEC is unclear but it has been recently shown that the relative number of MTECs correlates with the formation of aberrant crypt foci, that are well-recognized biomarkers of neoplastic transformation in the colon (15). Thus, it has been proposed that MTEC may be used as a relevant murine bioassay to study early stage colorectal tumorigenesis (15).

We propose that MTEC may be less prone to developing cancer than crypts that do not overexpress MTs, since MT overexpression is thought to be a protective mechanism against both the inflammation-related tissue damage (16) and the cytotoxicity of carcinogens (17). Overexpression of MTEC may prevent further mutations that could eventually lead to the neoplastic phenotype. This view agrees with the observation of a positive association between increased MT expression and a better prognosis of colonic cancer (14) and with the fact that negative-

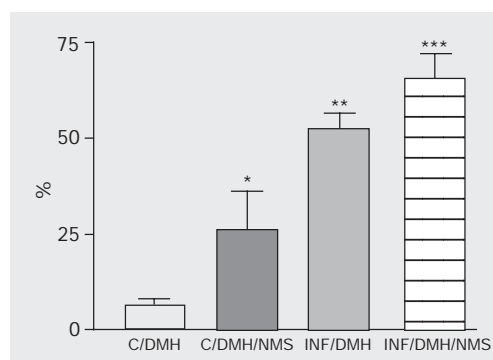
MT subclones may be present in colonic cancers, being responsible for a higher metastatic potential (14). Interestingly, it has been proposed that the initial driving forces behind environmentally induced cancers may not be mutations, but may rather be events related to carcinogen-induced selection through the epigenetic changes that eventually establish a gene expression program characteristic of a cancer cell (18).

The mechanisms of the increase in the number of MTEC are unknown. Crypt fission may explain the spread of mutated clones on the epithelial sheets (19). This proposed mechanism is related to colonic carcinogenesis by a process called field cancerization (20). The term field cancerization has been used to describe the multiple patches of the pre-malignant disease in the epithelial sheets that may explain the presence of synchronous distant tumors within one organ such as the colon (20).

On the basis of these considerations, we propose that the spread of an increased number of MTEC could play a role in the protection against cancer by nimesulide and by *T. cruzi* infection. This proposal is supported by the observation that MT is expressed in a patchy pattern in the neoplastic gastrointestinal tissues, with positive parts located next to negative parts (13), and that there is a clonal variation of the cadmium response in human tumor cell lines that may be related to the MT expression (21). Further studies are needed to confirm and further define the relationship between the spread of MTEC and its role in colonic carcinogenesis in rats treated with nimesulide or infected with *T. cruzi*.

The mechanisms involved in the formation of MTEC also need to be further elucidated. The colonic crypt can be considered to consist of a group of clonal cells, because each one of its cells arises from a single stem cell (9,11). It is known that the expression of the cell molecules may be influenced by epigenetic mechanisms during colonic car-

Figure 2. Metallothionein-positive crypt index of *Trypanosoma cruzi*-infected rats treated with dimethylhydrazine and nimesulide. INF/DMH/NMS - animals infected with *T. cruzi* and treated with nimesulide. INF/DMH - animals infected with *T. cruzi* and treated with dimethylhydrazine. C/DMH/NMS - animals treated with dimethylhydrazine and nimesulide; C/DMH - control animals treated with dimethylhydrazine. *C/DMH/NMS > C/DMH (P < 0.01, Kruskal-Wallis test); **INF/DMH > C/DMH/NMS > C/DMH (P < 0.01, Kruskal-Wallis test); ***INF/DMH/NMS > INF/DMH > C/DMH/NMS > C/DMH (P < 0.01, Kruskal-Wallis test).



cinogenesis (22). Recently, it has been observed that exposure to a non-genotoxic diet-related compound modulates the chemical carcinogenic effects of nitroso compounds for the induction of crypt-restricted MT immunopositivity (12). Furthermore, it has been proposed that the initial events in the environmentally induced cancers may be related to gene induction by epigenetic DNA methylation that leads to cancer cell selection and that the determination of the extent to which this concept applies to other environmental carcinogens is the challenge of

the future (18). We suggest that, in our experimental model, MT overexpression may have arisen as an inherited clonal trait, perhaps through an epigenetic regulatory mechanism. This experimental model may be useful for further studies on the epigenetic mechanisms involved in carcinogenesis. More detailed studies are also warranted to explain the mechanisms and consequences of the increase in MT expression associated with nimesulide and *T. cruzi* infection during colonic carcinogenesis.

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