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Research Article

cDNA Microarray Analysis of Nitric Oxide Pathway in Monocytes of Mice Infected with *Trichinellaspiralis*

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Abstract

Trichinellosis is a parasitic disease transmitted to humans mainly through pork consumption. To explore the mechanisms underlying NO regulation during *T. spiralis* infection a cDNA microarray analysis (114 genes] was performed. The NO pathway gene expression profiles were compared between 10, 20 and 39 days after *T.spiralis* experimental infection of BALB/c mice. Out of 114 genes, 18 (15,8%] genes were present in non-infected and post-infected mice. The expression of Ilk and Mt2 genes was significantly up regulated 10 days post-infection, while the expression of Mt2 gene was significantly up regulated 20 days post-infection. Furthermore, the expression of Fos, Fth1, IL-1b and Nfkbia genes was significantly down regulated 10 days post-infection, while the expression of Cxcl2 gene was significantly down regulated 10 and 20 days post-infection. The present study lists the candidate genes of the NO signaling pathway that were commonly and differentially expressed between different time points of *T. spiralis* infection and sheds light to the complex host parasite interplay.

ABBREVIATIONS

T. spiralis: Trichinellaspiralis NBL: Newborn Larvae; NO: Nitric Oxide; Inos: Inducible Nitric Oxide Synthase; TNF-Alpha: Tumor Necrosis Factor Alpha; IFN-Γ:Interferon Gamma; IL-10: Interleukin 10; TGF-Beta: Transforming Growth Factor Beta; HSP70: Heat Shock Protein 70; RBC: Red Blood Cells; Ilk: Integrin Linked Kinase; Mt2: Metallothionein 2; Fos: FBJ Osteosarcoma Oncogene; Fth1: Ferritin Heavy Chain 1; IL-1b: Interleukin 1 Beta; Nfkbia: NF-Kappa-B Inhibitor Alpha; Cxcl2: C-X-C Motif Chemokine Ligand 2; Th1/Th2: T Helper 1/T Helper 2; Qrt-PCR: Quantitative Real Time Polymerase Chain Reaction

INTRODUCTION

Trichinellaspiralis (*T.spiralis*] is a nematode with cosmopolitan distribution. *T. spiralis* species has a high infectivity to swine and rats, while it also infects a great variety of hosts, including humans [1]. Transmission occurs through ingestion of meat harbouring the L1 larvae of the parasite. These larvae upon digestion are freed and penetrate the epithelium of the small intestine, undergo molting and reach adulthood in about 30h [2]. After reproduction, the first newborn larvae [NBL] are present 5 days post-infection [3]. The NBL disseminate through the blood and lymph circulation and settle in the striated muscles of the host, where they develop into L1 larvae and remain infective for years [4].

In the course of Trichinella infection, the epithelium of the small intestine, lymph and blood circulation, and finally, muscle cells are affected. The host immune response is provoked and the defense against the parasite is manifested at both enteral and parenteral phases of its life cycle, as it has been previously demonstrated in rats [5].

T. spiralis can act as a moderator of the host response both in vitro and in vivo by employing diverse mechanisms, which are not yet thoroughly clarified [6]. It is widely accepted that nematode parasites have targeted several parts of the host signaling pathways, allowing them to interfere with the transcription of immune response genes [7,8]. In this context,

host-parasite relationships in Trichinella infections could be related to different cell mediators, e.g. Nitric Oxide [NO].

NO is a versatile biological agent, which acts as both pro- and anti-inflammatory mediator [9]. Inducible nitric oxide synthase [iNOS] is synthesized by a number of cell types while the most known triggers for its expression are endotoxins and cytokines. The cytokines Tumor necrosis factor alpha [TNF-alpha] and Interferon gamma [IFN-γ] as well as some chemokines, are strong inducers of NO. Conversely, Interleukin 10[IL-10] and Transforming Growth factor beta [TGF-beta] downregulate NO production [10]. NO is responsible for modulating nearly all steps of innate and adaptive immunity and has been shown to exert protective and toxic effects on the host's immune system [11].

To date, there is only scarce data regarding the NO signaling pathway. To elucidate how the parasite modulates the NO signaling pathway, it is pivotal to identify which genes are involved in the process. cDNA microarray analysis allows the simultaneous measurement of the expression profile of a large number of genes [12]. Aim of this study was to determine the NO pathway gene expression profiles at 10, 20 and 39 days after *T. spiralis* experimental infection in BALB/c mice.

MATERIALS AND METHODS

Animals, *T. spiralis* strain maintenance and blood collection Twenty BALB/c mice were obtained from Hellenic Pasteur Institute and kept under standard conditions with free access to water and feed [standard laboratory mice diet]. Their condition was followed daily. *T. spiralis* strain of swine origin was maintained and passaged. Infection doses of 200 *T. spiralis* L1 larvae were prepared from mouse muscle and fifteen BALB/c mice were infected per os. Blood collection was performed on days 10, 20 and 39 post infection from 10mice at each time point -in order to duplicate sampling-and from 5 control animals. The mice were kept in a restrainer while maintaining temperature at 24 to 27°C. Local anesthetic cream was applied on the tail 20 min before the experiment. Thereafter, the tail was cleaned with absolute alcohol and a 23G needle was inserted into the vein. Blood was collected in an EDTA tube and pressure was applied to stop bleeding. Blood samples from five animals at each time point were fused [1 sample per group] in order to obtain the adequate number of white blood cells necessary for sufficient RNA quantity and quality isolation.

RNA isolation

Red blood cells [RBC] were removed from whole blood samples using a density gradient centrifugation medium [Histopaque-I077, Sigma-Aldrich, USA]. Blood was diluted 1:2 with PBS, layered on to the top of Histopaque-1077 and centrifuged at 400xg for 30 min at room temperature. The white blood cell layer was carefully collected, rinsed twice with PBS and counted using a haemocytometer. Total RNA was isolated from the white blood cells with the ArrayGrade Total RNA isolation kit [SABiosciences, USA] according to manufacturer's instructions with an adjustment of the volume of Lysis and Binding buffer to the number of cells. RNA band integrity and DNA contamination were checked by gel electrophoresis. The purity of RNA samples was determined based on the ratio of spectrophotometric absorbance of the sample at 260 nm to that of 280 nm [A260/A280] using the Quant-iT Assay kit [Invitrogen, USA]. Only RNA samples with A260/A280 ratio > 2.0 were used for further experiments. Samples were frozen at -80°C until use in gene expression experiments.

SuperArray analysis - gene expression profiling

Gene expression profiles were created using the GEArray Q series NO mouse signaling pathway microarray, which is spotted with 114 mouse transcripts specific to NO signaling pathway [GEArray OMM-062, SABiosciences, USA]. Total blood RNA [1 μg] was used as template to generate Biotin-16-dUTP-labeled cDNA probes with the TrueLAbeling AMP 2.0 kit [SABiosciences, USA], according to the manufacturer's instructions. The cDNA probes were denatured and hybridized overnight with the NO SuperArray membrane. The array membranes were washed and blocked and alkaline phosphatase conjugated streptavidin was allowed to bind. After the COP-Star substrate [SABiosciences, USA] incubation, the chemiluminescent array image was captured by the automated MF-ChemiBIS workstation [DNR Bio-Imaging Systems, Ltd, USA].

Data analysis

The analysis of each microarray was conducted by GEArray Expression Analysis Suite 2.0. The abundance of each transcript was normalized to housekeeping gene markers on the array. Each probe was assigned a call of expressed [present] or not expressed [absent] using GEArray Expression Analysis Suite 2.0 decision matrix. The fold change values for the differentially expressed genes were calculated from ratios of intensities between pair samples. Genes that displayed a fold change greater than 1.5 or less than 0.7 were considered as differentially expressed. The M-value [log, fold change] was also estimated for all significant fold changes. Statistical evaluation employing student t-test [$p < 0.05$] was performed with the Microcal Origin 7.5 software [Microcal Software, Northampton, Maine]. In addition, BRB array tools were used for gene annotation obtaining data from the Gene Expression Omnibus public archive at the National Center for Biotechnology Information.

RESULTS AND DISCUSSION

A total of 114 genes of the NO signaling pathway gene array were screened on various time points after infection with *T. spiralis* in mice using superarray Q series. cDNA microarray analysis revealed that from the 114 genes, 18 [15.8%] genes were expressed [present] in non-infected and infected mice. The number of expressed genes in control and infected mice [10, 20 and 39 days post infection] is displayed in Figure 1. Based on the evaluation criteria [≥ 1.5 or ≤ 0.7-fold], the analysis revealed a differential expression for 18 genes of the 114 spotted sequences as illustrated in the corresponding scatterplot graphs [Figure 2]. In each case gene expression was calculated by determining the ratio of fluorescence intensity [degree of hybridization] corresponding to a gene in cDNA from uninfected mice to the mean fluorescence intensity for the same gene in cRNA from two replicate infected samples and is expressed as fold change. The expression of Ilk and Mt2 genes was significantly up regulated 10 days post-infection, while the expression of Mt2

gene was furthermore significantly up regulated 20 days post-infection. The expression of Fos, Fth1, IL-1b and Nfkbia genes was significantly down regulated 10 post-infection, while the expression of Cxcl2 gene was significantly down regulated 10 and 20 days post-infection. All the down or upregulated genes were also statistically different [$p < 0.05$] among the control and the infected groups [Figure 2].

Trichinellosis caused by *T. spiralis* is a parasitic zoonosis with worldwide distribution, which impacts on the development of animal husbandry and food safety, and thus threatens human health [13]. The host immune response to *T. spiralis* includes both innate and specific components, and is characterized by eosinophilic inflammation, which becomes mast cell and T cell dependent as the infection advances [14]. Previous studies have observed that many factors, such as cytokines, dendritic cells and regulatory T cells play an important role in the regulation of the host immune repertoire [6]. It has also been demonstrated that in *T. spiralis* infection a wide range of inflammatory cytokines such as IL-1, IL-4, IL-6, IL- 8, IFN-γ and TNF-alpha are produced [15,16].

T. spiralis possesses the capacity to rearrange the host immune cell response. In detail, this nematode can direct the immune system towards a mixed T helper 1/T helper 2 [Th1/Th2] response with predominance of Th2 phenotype and it is implicated in dendritic cell maturation. Furthermore, T. spiraliselicits the regulatory arm of the immune response via T or B regulatory cells while inducing the alternatively activated macrophages [15]. The mechanisms that the parasite employs to exert its immune regulatory effects remain poorly defined.

Nematode parasites have been proposed to reprogram host genomic transcription through their secreted molecules [17]. A previous study of the cDNA microarray analysis of 1176 genes suggested that many genes associated with cell differentiation and proliferation, as well as immune responses and apoptosis are likely involved during the course of *T. spiralis* infection [18]. Additional surveys have confirmed that many signaling pathways are involved in those processes, such as the transforming growth factor TGF-beta pathway in cell cycle arrest and transformation [19], mitochondria-mediated and TNF-alpha signaling pathways in apoptosis [20] and finally myogenic regulatory factors in satellite cell activation and differentiation [21].

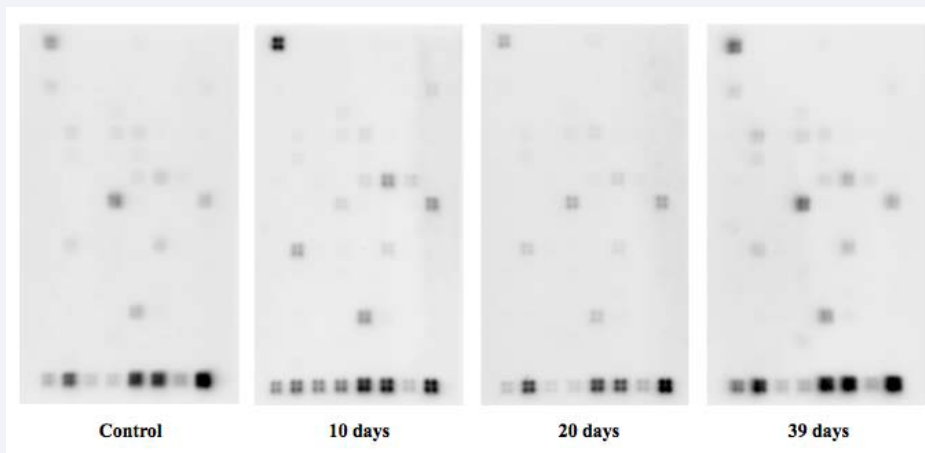

Figure 1 Microarray analysis of control and *T.spiralis* infected mice on days 10, 20 and 39 post infections. The four images correspond to the membranes used and illustrate the map of genes that are expressed (present) in control animals and on the three different time points of infection. Every expressed gene was identified as a specific hybridization signal that appeared as an image of tetra-spots. The presence of house keeping genes, which are expressed at the bottom line of the images with hybridization signals of various intensities, validates the method. In total 18 genes are expressed in non-infected and infected mice.

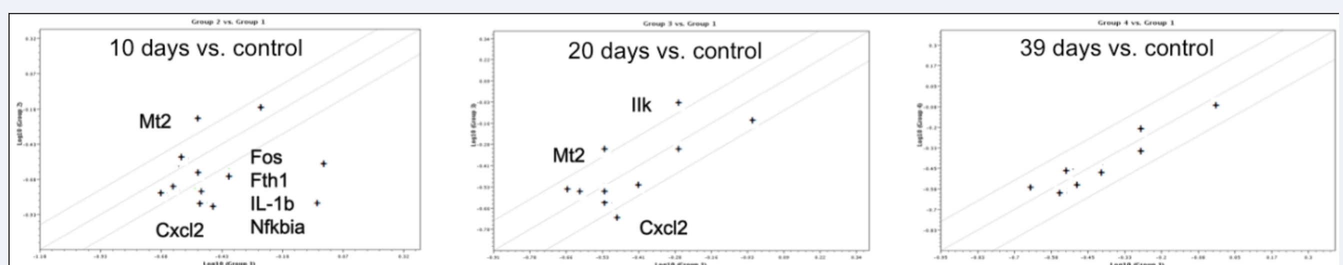

Figure 2 Scatterplot graphs illustrating the fold difference in the expression of genes in control and infected mice by relative expression levels among the control and the i) day 10 group, ii) day 20 group and iii) day 39 group. Each symbol corresponds to a specific gene. If the fold increase is greater than 1.5, the genes are represented above the lines (Ilk, Mt2). In case the fold is less than 1.5 and greater than 0.7 in either direction, the genes are represented between the lines (11 genes, not named). The genes are represented below the lines when the fold decrease is lower than 0.7 (Fos, Fth1, IL-1b, Nfkbia, Cxcl2). Housekeeping, control, blank and absent genes were omitted and are not presented in the scatterplots.

There is no doubt that the protective immune responses againstT. spiralisare multifactorial; however, they necessarily involve final effector molecules, including NO and iNOS [22,23]. NO has been recognized as one of the most important mediators in the immune system. A variety of immune cells produce or respond to NO and induction of iNOS has been implicated in several immunologic inflammatory conditions. iNOS expression is upregulated by Th1 cytokines and inhibited by Th2 cytokines, while the outcome of its activation is the production of NO, which is rapidly oxidized to reactive nitrogen species that are detrimental in many processes. Consequently, NO and iNOS are considered critical signaling molecules for parasite immunopathology and therefore have been proposed as possible targets for vaccine and therapy [24,25].

In experimental trichinellosis it has been demonstrated that iNOS is active in the pathology of skeletal muscle tissue [23] and that it contributes substantially to the associated enteropathy [26]. In addition, studies have demonstrated that encapsulated and non-encapsulated Trichinella species have the capacity to stimulate the secretion of NO from host macrophages [27]. It has been speculated that the protective effect of HSP70 [Heat Shock protein 70] during *T. spiralis* infection may be linked to stimulation of NO signaling pathways [28]. Moreover, it has been previously demonstrated that *T. spiralis* infection inhibits iNOS gene transcription, protein expression, and enzyme activity in many organs, such as the small and large intestine, kidney, lung, and uterus. The effect of this inhibition can overrule endotoxin-induced iNOS expression and may involve substances other than stress-induced corticosteroids [29].

In this study out of 114 genes of the NO signalling pathway, eighteen genes [15.8%] were present in non-infected and post-infected mice and need to be further investigated. The expression of Ilk and Mt2 genes was significantly upregulated post infection, while the expression of Fos, Fth1, IL-1b, Nfkbia and Cxcl2 genes was significantly downregulated post-infection.Up or down regulation of these genes implies that the amount of their corresponding encoded proteins is increasing or decreasing, respectively. Several of these differentially expressed genes encode proteins that are implicated in the regulation of diverse processes, such as cell cycle and differentiation, angiogenesis, muscle development, and apoptosis [30,31,32]. Furthermore, the encoded proteins constitute molecules of a complex network that coordinates NO production and functions. Consequently, elevation or depression of these proteins has an impact on NO production as well as on the host parasite relationship, which favors T. spiralis. Future research should focus on verifying the differential expression of the fore mentioned genes at a post-transcriptional level by quantitative real time Polymerase Chain Reaction [qRT-PCR]. The information obtained will be useful for exploring new strategies to effectively control NO production during trichinellosis. Nowadays a growing body of evidence sheds light on the nematode-derived mediators responsible for reprogramming the signaling pathways of the host and thus affect the pathogenesis of trichinellosis at the molecular level [33].

CONCLUSION

In conclusion, it all comes down to the complex host – parasite interaction. The current study deals with the profiles of genes of the NO signaling pathway, which are commonly and differentially expressed at specific points of *T. spiralis* infection and suggests that these genes need to be further investigated to identify the mechanisms underlying the complex cross-talk between parasite and host during trichinellosis. Although advances have been made, the regulation and relative importance of the individual *T. spiralis* modulation mechanisms of the host's signaling pathways still awaits further clarification. This could provide insights into the pathogenesis of trichinellosis and lead to the development of new biomarkers for diagnosis or treatment.

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