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Trichinella spiralis muscle larvae excretory–secretory products induce changes in cytoskeletal and myogenic transcription factors in primary myoblast cultures

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ABSTRACT

Trichinella spiralis infection in skeletal muscle culminates with nurse cell formation. The participation of excretory–secretory products of the muscle larvae has been implicated in this process through different studies performed in infected muscle and the muscle cell line C2C12. In this work, we developed primary myoblast cultures to analyse the changes induced by excretory–secretory products of the muscle larvae in muscle cells. Microarray analyses revealed expression changes in muscle cell differentiation, proliferation, cytoskeleton organisation, cell motion, transcription, cell cycle, apoptosis and signalling pathways such as MAPK, Jak-STAT, Wnt and PI3K-Akt. Some of these changes were further evaluated by other methodologies such as quantitative real-time PCR (qRT-PCR) and western blot, confirming that ML-ESP treated primary mouse myoblasts undergo increased proliferation, decreased expression of MHC and up-regulation of α-actin. In addition, changes in relevant muscle transcription factors (*Pax7*, *Myf5* and *Mef2c*) were observed. Taken together, these results provide new information about how *T. spiralis* could alter the normal process of skeletal muscle repair after ML invasion to accomplish nurse cell formation.

1. Introduction

Trichinella spiralis is an intracellular parasite of mammalian species. After a short intestinal phase, the newborn larvae (NBL) invade the skeletal muscle where they mature into muscle larvae (ML). During this process, *T. spiralis* triggers a series of changes in the infected muscle, which eventually lead to the transformation of the infected cell into the nurse cell phenotype (Teppema et al., 1973; Despommier, 1993). The nurse cell is a structure surrounded by a highly vascularized collagen capsule, which allows parasite survival for long periods of time until the parasite infects a new host (Baruch and Despommier, 1991; Wranicz et al., 1996; Despommier, 1998).

The multiple changes that ML infection elicits in skeletal muscle include the loss of contractile structures, nuclei hypertrophy, mitochondrial damage, basophilic transformation, apoptosis modulation and cell cycle re-entry followed by the arrest in G2/M phase (Despommier, 1975; Jasmer, 1993; Wranicz et al., 1996, 1998; Matsuo et al., 2000; Boonmars et al., 2004; Babal et al., 2011). These changes have been catalogued as a de-differentiation process followed by re-differentiation into an entirely different phenotype (Ko et al., 1994; Wu et al., 2008b). The nature of these events raises questions, and their solutions would provide new insights into new therapeutic approaches against this parasite.

Different approaches have been used to study the muscle alterations induced by *T. spiralis* infection, including microarray analysis of infected muscle (Wu et al., 2005, 2008a) and gene expression changes in C2C12 cells (Bai et al., 2012). Differential gene expression is observed in muscle transcription factors (*MyoD*, *MRF4*, *MyoG*, *MEF2c*) and genes related to satellite cell activation (*Pax3*, *Pax7* and *Desmin*), proliferation (*Pcna*, *Srib*), cell cycle (*p21*, *Rb1*) and apoptosis (*p53*, *MDM2*) (Wu et al., 2001, 2005, 2008a). In vitro, C2C12 cell treatment with excretory–secretory products (ESP) of

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the ML (ML-ESP) reduces muscle differentiation, induces proliferation and reduces the levels of *MyoD*, *Myogenin* and *p21* in vitro (Bai et al., 2012), which suggest that ML-ESP play a fundamental role in nurse cell formation.

ML-ESP are composed of a complex array of proteins, some of them tyvelosed (Wisnewski et al., 1993) and not fully characterised. MS analysis revealed multiple serin-proteases and a deoxyribonuclease II (Robinson and Connolly, 2005; Wang et al., 2013), while activity of serine/threonine protein kinase, metalloprotease, endonuclease, nucleoside diphosphate kinase and serine protease (Arden et al., 1997; Mak and Ko, 1999; Gounaris et al., 2001; Lun et al., 2003; Park et al., 2016) have also been reported.

Different studies have also detected some of these proteins in nuclei and cytoplasm of infected muscle (Despommier et al., 1990; Lee et al., 1991; Vassilatis et al., 1992; Boonmars et al., 2005; Guiliano et al., 2009). Muscle modifications induced by injection of ML-ESP are similar to those produced during ML development, which suggests that *T. spiralis* uses ESP to induce the necessary changes in muscle to allow nurse cell development (Ko et al., 1994).

The process of nurse cell formation shares some similarities to muscle repair (Matsuo et al., 2000; Wu et al., 2001, 2008b), since *T. spiralis* infection also causes the activation and proliferation of satellite cells but inhibits differentiation, altering the normal muscle repairing course to accomplish cystogenesis (Wu et al., 2008b). Therefore, it would be relevant to further study the changes occurring during this process. Until now, expression analysis has been performed in infected muscle (Wu et al., 2005, 2008a), and these tissues carry a variety of contaminating infiltrated cell types. In addition, studies in the C2C12 cell line may contain some genetic inaccuracies due to genetic and phenotypic differences from the original tissue (Bai et al., 2012).

Although the analysis of primary muscle cell cultures treated with ML-ESP was previously reported (Leung et al., 1997), only a morphological description was performed. Also, microarray analyses from *T. spiralis*-infected tissue late in the infection have already been reported (Wu et al., 2005, 2008a). Therefore, in this work, we developed a microarray expression analysis in primary myoblast cultures, in an attempt to elucidate the early changes induced in ML-ESP treated muscle cells that could lead to nurse cell formation. We found alterations in cytoskeleton organisation associated with changes in Myosin Heavy Chain (MHC) and α-actin protein and gene expression, presumably related to the morphological and some structural alterations in ML-ESP treated myoblasts. Furthermore, ML-ESP treatment produced changes in muscle transcription factors such as *Pax7*, *Myf5* and *Mef2c* which may give new insights into the mechanisms used by *T. spiralis* to accomplish cystogenesis.

2. Materials and methods

2.1. Animal source

Mice were obtained from the Institutional Animal Care and Use Committee (IACUC) of the Center for Research and Advanced Studies (IACUC–CINVESTAV), Mexico. Animal handling and all experimental protocols were fully accredited and performed in accordance with the Ethical Guidelines and Procedures from the IACUC–CINVESTAV, protocol number 0084-14, which fulfils the Mexican Official Norm (NOM-062ZOO-1999) "Technical specifications for the Care and Use of Laboratory Animals" and the National Institutes of Health, USA, guide for the care and use of laboratory animals.

2.2. ML-ESP preparation

ML-ESP were collected from *T. spiralis* muscle larvae (MSUS/MEX/91/CM-91) from experimentally infected CD-1 mice at least 30 days p.i. as previously described (Gamble et al., 1988; Appleton and Usack, 1993). Briefly, ML recovered from infected muscle were subjected to an acid-pepsin digestion, and then incubated in serum-free RPMI 1640 medium containing 100 U/ml of penicillin and 100 μg/ml of streptomycin, at 37 °C under 5% atmospheric CO_2. After 72 h, the supernatant was collected, filtered through a 0.2 μm pore-size membrane, precipitated with ammonium sulphate and dialyzed against PBS (1×). Protein concentration was determined by Bradford assay and integrity was evaluated by SDS–PAGE. ML-ESP was fractionated and kept at −70 °C.

2.3. Primary myoblast culture

Skeletal muscle was obtained from hind limbs of 2–4 days postnatal BALB/c mice. Digestion was performed at 32 °C as previously reported (Das et al., 2007; Sander et al., 2013). Cells were cultured at 37 °C and 5% CO_2 under proliferation conditions (Minimum Essential Medium Eagle, 10% FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin, MEM amino acids solution (Sigma (USA), M5550)) for 24 h and then induced to differentiate by cultivating in differentiation medium (DM) (DMEM supplemented with 3% FBS, 7% Adult Bovine Serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, MEM amino acids solution (Sigma, M5550) and GlutaMAX (Gibco, (USA) Cat. 35050061)). Depending on the assay, cells were grown for various periods of time in the presence of different ML-ESP concentrations. All experiments were performed from newly isolated cells with no more than 9 days under culture, without passages.

2.4. RNA isolation

Primary myoblasts (5×10^5 cells) were seeded onto 6 well plates. After a 24 h proliferation period, cells were induced to differentiate, and 50 μg of ML-ESP were added to the cultures at days 0 and 4 under differentiation conditions. After 8 days of ML-ESP treatment, total RNA was extracted from cells with Trizol (Invitrogen, USA), according to the manufacturer's instructions and digested with DNase I (New England Biolabs, USA). Concentration and purity of RNA were analysed by NanoDrop ND-2000 spectrophotometer (Thermo Scientific, USA) and used both in microarray analysis and quantitative real-time PCR (qRT-PCR) assays.

2.5. Microarray analysis

Total RNA extracted from cells was used for cDNA synthesis, sample labelling and hybridisation by the DNA Microarray Unit of the Institute of Cellular Physiology of National Autonomous University of Mexico (UNAM, Mexico). Microarray data analysis was performed using genArise software, developed by the Computing Unit of the Institute of Cellular Physiology of UNAM (http://www.ifc.unam.mx/genarise/). GenArise implements background correction, lowers normalisation, and intensity filter. It also replicates analysis and selects differentially expressed genes. This software identifies differentially expressed genes by calculating an intensity-dependent *z*-score with a sliding window algorithm, which calculates the mean and S.D. within a window surrounding each data point, and defines a *z*-score where *z* measures the number of S.Ds. that separates a data point from the mean.

$$z_i = [R_i\text{-mean}(R)]/\text{sd}(R)$$

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