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The promoter region of interferon-gamma is hypermethylated in neonatal foals and its demethylation is associated with increased gene expression

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ABSTRACT

While born with a limited production, foals' interferon-gamma (IFN-γ) expression increases after birth. The underlying mechanisms remain unknown. DNA methylation is considered to be involved. Therefore, the DNA methylation status of the *Ifng* promoter in $CD4^+$ cells from neonatal foal was determined using a methylation-specific PCR (MSP), and its relevance to IFN-γ mRNA expression was estimated. The effect of environment on the DNA methylation was also evaluated by comparing ponies that were kept in a barn versus those on pasture. The DNA in the *Ifng* promoter was hypermethylated and its demethylation was correlated with an increase in IFN-γ mRNA expression and age. This age-associated demethylation was accelerated by barn-air exposure. In conclusion, IFN-γ expression in foals appears to be controlled by DNA methylation in the promoter region of *Ifng*. The age-associated demethylation of the DNA in foals may be induced by exposure to environmental antigens and their effect on lymphoproliferation.

1. Introduction

Interferon-gamma (IFN-γ) expression is reduced in neonates of most species (Vuillermin et al., 2009), including foals (Breathnach et al., 2006). This reduced expression is associated with an increased risk for intracellular bacterial infections, such as those caused by *Rhodococcus equi* (*R. equi*). This depressed IFN-γ expression in the neonate is likely the consequence of a protective strategy against fetal loss caused by IFN-γ production at the fetal/maternal interface (Murphy et al., 2009). The expression of IFN-γ mRNA correlates with protein production in foals indicating its expression is regulated at the transcriptional level (Breathnach et al., 2006). However, the underlying regulatory mechanisms are unknown. DNA methylation, induced by DNA methyltransferase (DNMT), is widely accepted as a primary mechanism for regulating gene transcription (Ansel et al., 2003; Holliday, 2006). Methylation inhibits gene transcription either directly by inhibiting transcription factor (TF) binding or by allowing a methyl-binding protein to bind to the methylated DNA which ultimately "closes" chromatin structure (Spilianakis and Flavell, 2007). In the case of humans and mice, the degree of methylation of the promoter determines the level of IFN-γ expression (Spilianakis and Flavell, 2007; Wilson et al., 2009). Thus, hypermethylation of the CpG motif in the *Ifng* promoter region results in limited IFN-γ expression in human neonates (White et al., 2002). Reduced IFN-γ expression in neonatal foals may likewise be the result of DNA hypermethylation.

In foals, as in other neonates, IFN-γ expression increases rapidly after birth (Breathnach et al., 2006). This temporal increase in IFN-γ expression is accelerated by exposure to environments containing high levels of bacterial and fungal antigens (Sun et al., 2011). While it is postulated that DNA methylation of *Ifng* may be altered by environmental exposure to microbial antigens, there is little evidence available to support this hypothesis (Vuillermin et al., 2009). Therefore, we examined the methylation status of the promoter region of *Ifng* in the foal in order to correlate methylation status with *Ifng* expression. We also determined the effect of the environmental exposure to microbial antigens on the methylation status of the foals' *Ifng* promoter. Lastly, we determined the effect of the environment on lymphoproliferation since proliferation is reported to regulate DNA demethylation (Wilson et al., 2009).

Abbreviations: IFN-γ, interferon-gamma; PCR, polymerase chain reaction; MSP, methylation specific PCR; PBMC, peripheral blood mononuclear cells; ConA, concanavalin A; ^{m}CpG, methylated CpG; ^{u}CpG, unmethylated CpG; DNMT, DNA methyltransferase.

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2. Materials and methods

2.1. Horses

A group of 12 horse foals (2–16 weeks old) and four adult horses (6–10 years old) were used to determine DNA methylation level in the equine IFN-γ promoter. The horses were housed at the Department of Veterinary Science's equine facility in Lexington, Kentucky. Another group of 10 pony foals were used for the study of environmental effects on the DNA methylation, along with 2 adult ponies (6 and 8 years old). The foals were maintained on the University of Kentucky's Department of Veterinary Science's farm in Versailles, Kentucky. Five of the pony foals were chosen randomly at birth to spend 4 h a day for 3 days (MWF) per week in individual stalls with their mares. This barn exposure started when the foals were less than 1 week old and stopped after they reached 2 months of age. When not in the barn, the foals were kept on pasture with their mares. Throughout the study period, the ponies, as well as the horses above, had ad libitum access to water and forage in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research. All research procedures were approved by the University of Kentucky's Institutional Animal Care and Use Committee.

2.2. Lymphocytes isolation and cell sorting

Heparinized blood was collected from each horse by aseptic jugular venipuncture. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation, as previously described (Breathnach et al., 2006). The cells were resuspended in cRPMI 1640 media (Gibco, Grand Island, NY) supplemented with 2.5% fetal equine serum (FES; Sigma, St. Louis, MO), 2 mM glutamine (Sigma), 100 U/ml penicillin/streptomycin (Sigma), 55 mM 2-mercaptoethanol (GIBCO) and surface stained for CD4, as described (Merant et al., 2009). The cells were labeled with primary antibodies to CD4 (CVS4) or an IgG1 isotype control (BD Pharmingen™, Sparks, MD) followed by fluorescent labeling with secondary antibodies (PE conjugated goat Fab' anti-mouse IgG [Southern Biotechnology Associates, Birmingham, AL]). The $CD4^+$ cells were then sorted by a Cytomation MoFlo® high-speed cytometer cell sorter and analyzer from Cytomation (Fort Collins, CO) as gated (Supplementary Fig).

2.3. Quantitation of cellular proliferation and IFN-γ expression

Proliferation was assessed using flow cytometry, as previously described (Sun et al., 2012). Briefly, 1×10^7 PBMC were suspended in 1 ml PBS (Sigma) and stained with 1 ml of carboxyfluorescein succinimidyl ester (CFSE; 5 μM/ml, Sigma) for 8 min. The reaction was quenched by adding 2 ml of FBS (Sigma), followed by two washes with PBS supplemented with 10% FBS. The cell pellet was then resuspended in 5 ml of cRPMI media (final concentration of 2×10^6 cells/ml). The CFSE – labeled PBMC were transferred to a 24-well-plate at 1 ml/well. The cells were then stimulated with 3 μg/ml concanavalin A (ConA; Sigma) for 4 days. Afterwards, the cells were washed with FACS Flow and analyzed with a FACSCalibur (BD, Franklin Lakes, NJ) flow cytometer. The Proliferation Index (PI) was calculated using ModFit LTTM (Version 3.0, Verity Software House, Inc., Topsham, ME).

For IFN-γ analysis, ConA-stimulated PBMC were pulsed with phorbol 12-myristate 13-acetate (PMA; 25 μg/ml; Sigma, p8139) /ionomycin (iono; 1 μM; Sigma) and brefeldin A (BFA; 10 μg/ml; Sigma) during the last 4 h of incubation. Intracellular staining for IFN-γ was performed, as previously described (Sun et al., 2012).

2.4. RT-PCR

Total cellular RNA was isolated from 1×10^6 cells preserved in RNA-STAT 60 (Tel-Test, Inc. Friendswood, TX) according to the manufacturer's protocol. Approximately 3.0 μg of total RNA was isolated from the cells. Reverse transcription was performed as described previously (Breathnach et al., 2006). Briefly, 0.5 μg of RNA was suspended in 49.5 μl of nuclease-free water (Qiagen, Valencia, CA) and added to 30.5 μl reverse transcription master mix (0.5 ml [20 U/ml] avian myeloblastosis virus [AMV] reverse transcriptase [Promega, Madison, WI], 1 ml oligo dT primer [0.5 mg/ml; Promega], 1 μl RNAsin [40 U/ml, Promega], 4 μl dNTP [10 mM; Promega], 8 μl AMV buffer [Promega], and 16 μl $MgCl_2$ [25 mM, Promega]) was added to each sample. A RT-negative control was also included. The reactions were incubated at 42 °C for 15 min and 95 °C for 5 min in a thermocycler. The resulting cDNA was diluted 1:1 with RNAse-free water. The gene expression was quantitated using an Applied Biosystems PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Intron-spanning equine IFN-γ primers and probe failed to amplify genomic DNA and reverse transcription negative RNA samples and their amplification efficiencies were greater than 95%, as determined using LinRegPCR (Ramakers et al., 2003). PCR reactions were performed in duplicate wells per sample, as described previously (Breathnach et al., 2006). Beta-glucuronidase (b-Gus) was used as the housekeeping gene and the relative quantification (RQ) method for mRNA expression was used (Breathnach et al., 2006).

2.5. Methylation specific PCR (MSP)

The likely promoter region of equine *Ifng* was predicted using promoter 2.0 prediction server (http://www.cbs.dtu.dk/services/Promoter/). Based on the CpG sites, three sets of MSP primers were designed by Methprimer (http://www.urogene.org/methprimer/index1.html) and synthesized by Integrated DNA Technologies, Inc. (IDT, Coralville, IA). The primers sequences are listed in Table 1.

Genomic DNA was isolated using Gentra Puregene Cell Kit (QIAGEN, Valencia, CA) and 400 ng of the DNA was bisulfite treated using EZ DNA Methylation-Gold™ Kit (D5005, Zymo Research Corporation, Irvine, CA). The real time PCR was performed in duplicate for each sample with 2 mM of specific primers for methylated CpG (^{m}CpG) or un-methylated CpG (^{u}CpG), 30 ng of bisulfite converted genomic DNA as template, and 10 μl of PCR reaction mix (QuantiTec® SYBR® Green PCR Kit, QIAGEN), in a total of 20 μl. The PCR reaction conditions were as follows: pre-heat at 95 °C for 15 min; 10 cycles of touch-down PCR with 95 °C 30 s, 60 °C 30 s (−0.5 °C per cycle) and 72 °C 30 s; 40 cycles of amplification with 95 °C 30 s, 55 °C 30 s, 72 °C 30 s, 60 °C 45 s (signaling); 72 °C 10 min for final elongation followed by determination of dissociation curve. Completely methylated genomic DNA generated by methyltransferase (M0226, New England Biolabs, Ipswich, MA) from genomic DNA was used as a positive control, and the completely un-methylated genomic DNA generated using GenomiPhi DNA Amplification Kit (25-6600-30, GE healthcare, Piscataway, NJ) was used as a negative control. The Ct values were corrected using LinRegPCR, with efficiencies below 90% excluded. The percentage of CpG methylation for each region of *Ifng* promoter was calculated as $^{m}\text{CpG}\% = (2^{-\text{CtmCpG}}/(2^{-\text{CtmCpG}} + 2^{-\text{CtuCpG}})) \times 100\%$.

2.6. Total DNMT activity test

Nuclear protein was extracted from $CD4^+$ T cells using EpiQuik™ Nuclear Extraction Kit I (P-0002, Epigentek Group Inc., Farmingdale, NY). The protein was aliquoted and stored at −80 °C. The concentration of the nuclear protein was determined using a BCA protein Assay Kit (23225, Fisher Thermo Scientific