

Osteopontin Expression in Periparturient Dairy Cows Naturally Infected with *Mycobacterium Avium* Subsp. *Paratuberculosis*

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Summary and Implications

Twenty-five multiparous Holstein cows were grouped according to infection status with *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the causative microorganism of Johne's disease (JD). Osteopontin (Opn) was characterized at both the level of gene and protein expression. Results of this study indicate that in dairy cows, expression of Opn is modulated by natural infection with MAP and by the periparturient period.

Introduction

Johne's disease (JD), caused by MAP, is estimated to infect more than 22% of US dairy herds and cost the US dairy industry in excess of \$200 million annually. Dairy cows generally are infected as neonates by the ingestion of feed or milk contaminated with the organism. Once infected, animals may remain in the subclinical, or asymptomatic stage of the disease until a period of stress, such as parturition, occurs. Osteopontin is a highly acidic glycoprotein that is produced by both immune and non-immune cells. The primary immune sources of Opn are activated macrophages, activated T-cells, and dendritic cells. The role of Opn in mycobacterial infection is of interest based upon its reported ability to upregulate and promote pro-inflammatory cytokines. An effective Th1 response to MAP infection is critical for controlling the initial stages of the disease. The transition from subclinical to clinical stage of disease coincides with a shift from Th1- to Th2-mediated host responses. To date, there are no reports in the literature of Opn expression in dairy cows infected with MAP. Based upon this observation and the critical role of Opn in controlling mycobacterial infections, the objective of this study was to characterize Opn at both the level of gene and protein expression in periparturient dairy cows naturally infected with MAP.

Materials and Methods

Twenty-five multiparous Holstein cows were grouped according to infection status. These three groups consisted of 8 noninfected healthy cows, 10 cows naturally infected with MAP, but asymptomatic, and 7 naturally infected cows with clinical JD. Animals were categorized by historical monitoring for fecal shedding of bacteria, gamma interferon

(IFN- γ) expression, and MAP antibody titer. Blood was collected from the jugular vein at -21, -14, -7, +1, +7, +14, +21, +28, and +35 days relative to calving. Peripheral blood mononuclear cells (PBMC) were isolated and cultured in RPMI for 24 hours at 39°C, under 5% CO₂, and in 75-cm² flasks. Cells were not stimulated (NS) or stimulated with MAP whole cell sonicate (MPS; 10 μ g/mL). RNA was extracted using TriZol reagent, purified using the RNeasy® Mini Kit protocol for RNA cleanup, and converted into first strand cDNA. Real time-PCR then was performed using the Applied Systems 7500 DNA sequence detection system to evaluate the expression of Opn. RT-PCR data were analyzed using the 2^{-(ddCt)} method. For immunoblot analysis, total protein was extracted from PBMCs and anti-mouse Opn antibody was used to detect the presence of Opn protein in the lysates. Digital images of the autoradiographic film from the immunoblots were scanned, and scanning densitometry analysis was performed. Data were analyzed using PROC MIXED analysis of SAS. Means differed if $P < 0.05$ and tended to differ if $0.05 \leq P \leq 0.15$.

Results and Discussion

The ability of Opn to upregulate and promote pro-inflammatory cytokines in response to intracellular bacterial infections suggests it may have a profound effect on MAP-infected dairy cows. Overall, expression of Opn from NS PBMCs was greater ($P < 0.05$) in subclinically infected animals compared with control and clinical cows (Figure 1). This is important because an increase in Opn expression supports the Th1 host response typically observed in subclinically infected dairy cows. For the subclinical cows, Opn expression increased during the postpartum period and was significantly greater ($P < 0.05$) at +14, +28, and +35 d compared with +1 d. These data are consistent with the established increase in the Th1 immune response that occurs in periparturient dairy cows during the postpartum period. Osteopontin expression in NS PBMCs was reduced 7.7-fold between -21 and +1 d ($P < 0.001$) in control cows and 6.9-fold between -7 d and +1 d ($P < 0.01$) in clinical cows. These data are consistent with the established decrease in the Th1 immune response that occurs in periparturient dairy cows during the prepartum period.

When PBMCs were stimulated with MPS, control cows expressed more Opn than subclinical ($P < 0.001$) and clinical ($P < 0.05$) cows did (Figure 2). For subclinical cows, expression increased ($P < 0.001$) at calving and then declined during the postpartum period. In contrast, a decline ($P < 0.03$) in Opn expression by MPS-stimulated PBMCs was observed for clinically infected cows as calving approached, followed by an increase during the postpartum period.

This was the first study to report protein expression of Opn in PBMCs isolated from Holstein dairy cows. The immunoblotting procedure detected Opn protein bands at 24, 37, 50, and 62-kDa in the PBMC lysates of all animals (Figure 3). Purified bovine milk Opn was used as a standard and identified as a single band at 60 kDa (data not shown). Multiple isomers of the Opn protein are not uncommon in tissues. The densities of the 24, 37, and 62-kDa proteins varied extensively between cows over the periparturient period and the variation did not seem related to infection group. The 50-kDa protein was consistently the most intense of the four detected isomers. Therefore, in order to determine the relative abundance of Opn, the 50-kDa band was analyzed. Osteopontin abundance increased in PBMCs isolated from control cows throughout the sampling period ($P < 0.02$) (Figure 4). PBMCs isolated from subclinical cows had an increase in protein abundance at calving and then declined during the postpartum period ($P < 0.06$).

This study presents the first known data examining Opn gene and protein expression in periparturient dairy cows naturally infected with MAP. Results of this study indicate that in dairy cows expression of Opn is modulated by natural infection with MAP and by the periparturient period. Furthermore, the present study demonstrates for the first time the presence of Opn protein expression in PBMCs of healthy and MAP-infected dairy cows. Our data suggest that Opn may be a key regulator of MAP infection.

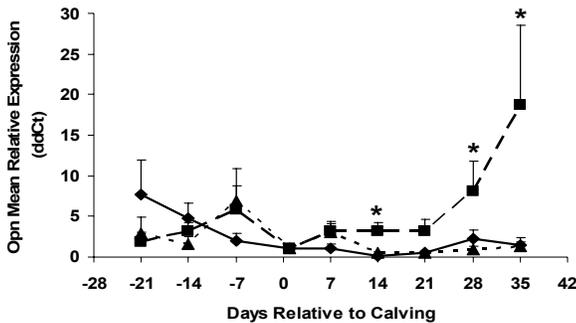


Figure 1. Osteopontin gene expression by non stimulation PBMCs isolated from whole blood of control (◆), subclinical (■), and clinical (▲) periparturient dairy cows. Infection group, $P < 0.05$; Infection group x Parturition, $P < 0.05$. Data are means \pm SE. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

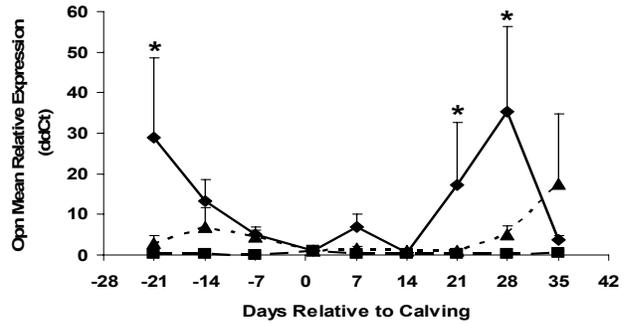


Figure 2. Osteopontin gene expression by MPS PBMCs isolated from whole blood of control (◆), subclinical (■), and clinical (▲) periparturient dairy cows. Infection group, $P < 0.05$. Data are means \pm SE. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

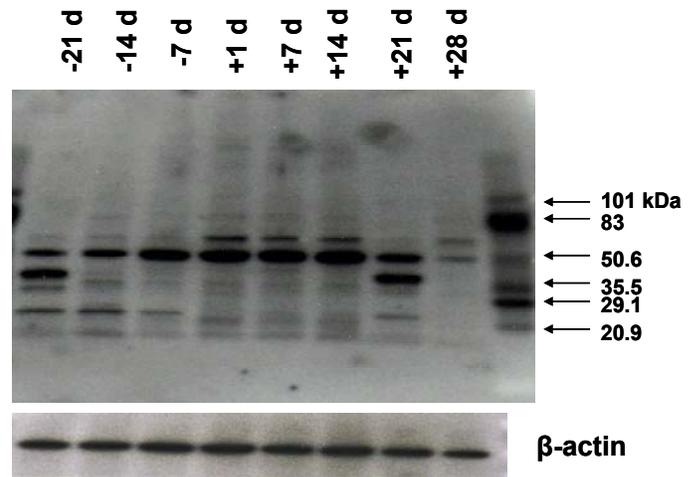


Figure 3. Detection of osteopontin protein using immunoblot analysis. Picture is of a representative blot from a subclinical MAP-infected periparturient dairy cow. Isomers were detected at 24, 37, 50, and 62-kDa.

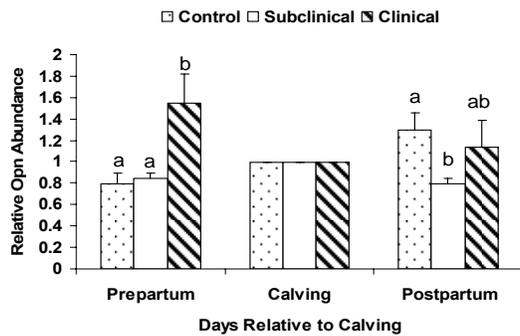


Figure 4. Osteopontin protein expression in PBMCs isolated from peripheral blood of control, sunclinical, and clinical periparturient dairy cows naturally infected with MAP. Densitometric analysis of Opn relative abundance compared with calving. Infection group x Parturition, $P < 0.01$. Data are means \pm SE. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).