J. Dairy Sci. 91:3079–3091
doi:10.3168/jds.2008-1061

Osteopontin: A Novel Cytokine Involved in the Regulation of *Mycobacterium avium* subspecies *paratuberculosis* Infection in Periparturient Dairy Cattle

E. L. Karcher,* D. O. Bayles,† J. P. Bannantine,† D. C. Beitz,* and J. R. Stabel†[1]
*Department of Animal Science, Iowa State University, Ames 50011
†USDA-ARS, National Animal Disease Center, Ames, IA 50010

ABSTRACT

Osteopontin (Opn), an important mediator of the cell-mediated immune response, enhances the host immune response against mycobacterial infections. Infections caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP) have a devastating effect on the dairy industry. We sought to characterize Opn at the level of gene and protein expression in periparturient dairy cows naturally infected with MAP. Peripheral blood mononuclear cells (PBMC) were isolated from control, subclinical, and clinical periparturient dairy cows naturally infected with MAP beginning 3 wk precalving to 5 wk postcalving and incubated with medium alone (nonstimulated: NS), concanavalin A (ConA), or a whole-cell sonicate of MAP (MPS). Real-time PCR was performed to evaluate expression of Opn and classical Th1 and Th2 cytokines. Results demonstrated greater Opn expression in nonstimulated PBMC isolated from subclinical cows compared with control and clinical cows. For clinical cows, there was a strong correlation between Opn expression and expression of the Th1 cytokines IFN-γ and IL-1α for nonstimulated PBMC and IFN-γ and IL-12 for PBMC stimulated with MPS. Expression of tumor necrosis factor-α was greater in clinical cows than the other groups. Nonstimulated, ConA, and MPS-stimulated PBMC from subclinical cows secreted more IFN-γ, and MPS-stimulated PBMC from clinical cows secreted more IL-4 compared with the other groups. Immunoblot analysis of PBMC detected 4 Opn proteins at 60, 52, 34, and 27 kDa. This is the first study to evaluate the role of Opn on the immune response of dairy cows naturally infected with MAP, and results suggest Opn may be a key regulator against MAP infection.

Key words: osteopontin, periparturient, *Mycobacterium avium* ssp. *paratuberculosis*

INTRODUCTION

The causative agent of Johne's disease (**JD**) in ruminant animals is *Mycobacterium avium* ssp. *paratuberculosis* (**MAP**). Dairy cows are generally infected as neonates by the ingestion of feed or water contaminated with the organism. Once they are infected, animals may remain in the subclinical or asymptomatic stage of disease until a period of stress, such as parturition, occurs. Advancement to the clinical stage of disease is characterized by fecal shedding of the bacteria, severe weight loss, and intermittent diarrhea. There are no known cures for JD, and often the animal will succumb to the infection.

Osteopontin (**Opn**), also identified as early T-cell activator-1, is a highly acidic glycoprotein that is produced by both immune and nonimmune cells, such as osteoclasts, smooth muscle cells, and epithelial cells (Denhardt and Guo, 1993). The primary immune sources of Opn are activated macrophages (Atkins et al., 1998), activated T cells (Ashkar et al., 2000), and dendritic cells (Kawamura et al., 2005). Osteopontin is also secreted from activated natural killer cells (Pollack et al., 1994), and it is an important modulator of the cell-mediated, or Th1, immune response. Osteopontin induces T-cell chemotaxis and costimulates T-cell proliferation (O'Regan et al., 2000). Stimulating murine macrophages with Opn resulted in the production of proinflammatory cytokines IL-12 and tumor necrosis factor-α (**TNF-α**; Weber et al., 2002). In addition, when human gut-derived T cells were stimulated with bovine Opn and CD3 antibody, there was a dose-dependent increase in the secretion of IFN-γ and TNF-α (Agnholt et al., 2007). In addition to its effects on T cells, Opn stimulates IgM and IgG production from B cells (Lampe et al., 1991).

The role of Opn in mycobacterial infection is interesting, because Opn is reported to upregulate and promote the expression and secretion of Th1 cytokines. When Opn knockout mice were challenged with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG Pasteur), they had more severe infection, heavier bacterial loads,

Received January 30, 2008.
Accepted April 6, 2008.
[1]Corresponding author: judy.stabel@ars.usda.gov.

and greater granuloma burdens compared with the wild-type mice (Nau et al., 1999). Infecting human alveolar macrophages with *Mycobacterium tuberculosis* was accompanied by an upregulation of Opn expression (Nau et al., 1997). Furthermore, an inverse correlation has been noted between the amount of Opn in tissues and disease severity in patients with mycobacterial infections (Nau et al., 2000). Patients suffering from nontuberculous mycobacterial infections with high Opn protein levels in the lymph nodes recovered faster compared with those patients with lower Opn levels (Nau et al., 2000).

The ability of Opn to promote a Th1 immune response and increase resistance to mycobacterial infections makes this cytokine important to study in MAP-infected cattle. An effective Th1 response to MAP infection is critical for controlling the initial stages of the disease. Subclinical JD cows produce greater amounts of the Th1 cytokines IFN-γ and TNF-α compared with clinical cows (Stabel, 2000). The transition from the subclinical to clinical stage of disease coincides with a shift from Th1- to Th2-mediated host responses. Production of Th2 cytokines supports a humoral immune response by stimulating the proliferation of B lymphocytes and inhibiting Th1 cytokines. Previous reports have demonstrated an upregulation of both IL-10 and transforming growth factor (**TGF**)-β in the tissues of clinical MAP-infected cows (Khalifeh and Stabel, 2004).

To date, there are no reports in the literature of Opn expression in dairy cows infected with MAP. Based on this observation, and the critical role of Opn in controlling other 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. In addition, the expression and secretion of other key Th1 and Th2 cytokines was performed during this time period and correlated with Opn results.

MATERIALS AND METHODS

Animals

Twenty-five multiparous Holstein cows were placed into 3 groups according to infection status. These 3 groups consisted of 8 noninfected healthy cows, 10 cows naturally infected with MAP but asymptomatic, and 7 naturally infected cows with clinical JD. The stage of infection was determined by fecal shedding of MAP, IFN-γ secretion, and specific antibody response to MAP. Infection was monitored by bacteriologic culture for the fecal shedding of MAP by standard methods (Stabel, 1997). By definition, clinical animals were shedding more than 100 cfu/tube of media (BBL Herrold's Egg Yolk Agar Slants with mycobactin J, 2 mg/mL; amphotericin, 50 μg/mL; nalidixic acid, 50 μg/mL; and vancomycin, 50 μg/mL; Becton, Dickinson and Co., Sparks, MD) and presented with weight loss and intermittent diarrhea. Subclinically infected cows were shedding less than 10 cfu/tube and were asymptomatic. The noninfected control cows were characterized by repeated negative fecal cultures performed quarterly over a 3- to 5-yr period and had been purchased from herds with no recent history of JD. In addition, these animals were negative on any serologic assays (i.e., production of antibody specific for MAP and IFN-γ) performed during that period. All procedures performed on the animals were approved by the Institutional Animal Care and Use Committee (National Animal Disease Center, Ames, IA).

Blood Collection, Culture Conditions, and Sample Collection

Blood was collected from the jugular vein in 2× acid-citrate-dextrose (1:10). For each animal, blood was collected at –21, –14, –7, +1, +7, +14, +21, +28, and +35 d relative to calving. Peripheral blood mononuclear cells (**PBMC**) were isolated from the buffy coat fractions of peripheral blood. The PBMC were resuspended in RPMI-1640 (Gibco, Grand Island, NY) with 10% fetal calf serum (Atlanta Biologics, Atlanta, GA), 100 U of penicillin G sodium per milliliter, 100 μg of streptomycin sulfate/mL, 0.25 μg of amphotericin B/mL, and 2 m*M* L-glutamine (Gibco). Cells were cultured at 1.4×10^6/mL in 48-well flat-bottomed plates (Corning, Corning, NY) with either medium alone (nonstimulated; NS), with concanavalin A (ConA; 10 μg/mL), or with MAP whole-cell sonicate (MPS;10 μg/mL) added to designated wells. Plates were incubated for 24 h at 39°C in 5% CO_2 in a humidified atmosphere. After 24 h, plates were centrifuged at $400 \times g$ for 5 min. Supernatants were removed without disturbing the cells in culture and stored at –20°C before cytokine measurement.

Bacteria

Mycobacterium avium ssp. *paratuberculosis* strain K-10 (National Animal Disease Center) was grown in Middlebrook 7H9 broth (pH 6.0) supplemented with mycobactin J (2 mg/L; Allied Monitor, Fayette, MO) and oleic acid-albumin-dextrose complex (Becton Dickinson Microbiology, San Jose, CA). The bacteria were harvested and washed 3 times with PBS (137 m*M* sodium chloride, 10 m*M* phosphate, 2.7 m*M* potassium chloride; pH 7.4). The MPS was then prepared by sonication of MAP (1×10^9/mL) in PBS at 25 W for 25 min on ice (Tekmar sonic disturber, Lorton, VA), and a protein concentration was determined. The MPS was diluted