Osteopontin immunoreactivity in the ileum and ileocecal lymph node of dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*

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Abstract

Osteopontin (Opn), a highly acidic glycoprotein, promotes cellular adhesion and recruitment and has been shown to be upregulated in the granulomas of mycobacterial infections. Johne’s disease, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is associated with granulomatous enteritis. The objective of this experiment was to identify Opn in the ileum and ileocecal lymph node (ICN) of dairy cows naturally infected with MAP and to compare the frequency and intensity of staining between noninfected healthy controls, subclinical and clinical cows. Sections from these three groups of animals were selected from a tissue archive. Immunohistochemical analysis was used to determine the location and expression of Opn. The frequency and intensity of staining was also reported. Confirmation of acid-fast bacilli in the tissue sections was achieved by the Ziehl-Neelsen method. Within the ileal tissue, macrophages, lymphocytes, and plasma cells stained positive for Opn. Clinical cows expressed Opn at a greater frequency in the lamina propria. Control and subclinical cows did not have areas of granulomatous inflammation but cells staining for Opn were equally intense for the three groups. The frequency of staining for Opn in the ICN was not affected by MAP infection. Results of this study confirm for the first time, the expression of Opn in the ileum and ICN of MAP-infected cattle. Published by Elsevier B.V.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*; Osteopontin; Granulomas

1. Introduction

*Mycobacterium avium* subsp. *paratuberculosis* (MAP), the causative agent of Johne’s disease, is currently a major economic and welfare issue for dairy producers in the United States. Once infected, animals may remain in the subclinical, or asymptomatic, stage of the disease for several years. The formation of granulomas at the site of MAP infection is critical for the early control of the infection (Coussens, 2001). In subclinical cows, well-demarcated, small granulomas located in the distal ileum and ICN were predominantly composed of macrophages, multinucleated Langherans giant cells, and few lymphocytes (Gonzalez et al., 2005). Lesions from clinically infected cows show diffuse granulomatous enteritis and consist primarily of lymphocytes, plasma cells, giant cells, and macrophages (Gonzalez et al., 2005).

Osteopontin (Opn) is a highly acidic glycoprotein that is produced by activated macrophages (Atkins et al., 1998), activated T-cells (Ashkar et al., 2000), and...
dendritic cells (Kawamura et al., 2005). The role of Opn in mycobacterial infections is of interest based upon its reported ability to promote the production of Th1 cytokines and to enhance host resistance against mycobacteria (Nau et al., 1999; Weber et al., 2002). Stimulating macrophages with Opn resulted in the production of the proinflammatory cytokines, IL-12 and TNF-α (Weber et al., 2002). The Th1 cytokines TNF-α and IFN-γ are essential for the production of the protective granulomas in MAP-infected cows (Roach et al., 2002).

Although Opn is constitutively expressed, it is upregulated in inflamed tissues, such as granulomas (Nau et al., 1997; O’Regan et al., 1999). Macrophages interact with Opn through the CD44 receptor and engagement induces chemotaxis and chemoattractant activity (Weber et al., 2002). This is important for the formation of granulomas, as the interaction facilitates the movement of macrophages and lymphocytes from peripheral blood to the tissue.

The objective of this study was to identify Opn in the ileum and ileocecal lymph node (ICN) of dairy cows naturally infected with MAP and to compare differences in immunoreactivity between the different infection groups. This is the first study to report Opn localization in the intestinal tract of MAP-infected cows and differences in Opn expression at the site of infection.

2. Materials and methods

2.1. Samples

This study was designed as a retrospective study in which sections were selected from an archive of tissue samples from healthy control dairy cows and cows with subclinical and clinical MAP infection. While in the herd, animals were routinely evaluated on a quarterly basis to determine infection status, which was monitored by culturing the feces for MAP using standard culture methods (Stabel, 1997). By definition, clinical animals were shedding more than 100 CFU/ tube of feces and presented with weight loss and intermittent diarrhea. The noninfected control cows were characterized by repeated negative fecal cultures performed quarterly over a 3- to 5-year period. In addition, the animals were negative on serologic assays (i.e., production of antibody specific for MAP and IFN-γ) performed during that period. Standard procedure in our laboratory mandates a complete necropsy of animals on-site after death, including tissue collection for histological and immunohistochemical examination. Ileum and ICN are two tissues routinely collected.

The number of ileal sections included samples from 4 controls, 4 subclinical, and 3 clinical cows. Sections of ICN were obtained from 5 control, 5 subclinical, and 5 clinical cows. When available, sections of ileal and ICN tissues were utilized from the same animals. Samples of tissues from all cows were fixed in neutral-buffered 10% zinc-formalin, processed routinely, and embedded in paraffin.

2.2. Immunohistochemistry

Sections were cut at 4 μm and fixed on ProbeOn™ Plus microscope slides (Fisher Scientific; Pittsburgh, PA). Sections were then deparaffinized in xylene and rehydrated by an ethanol series (100%, 95%, 70%, distilled water). An antigen retrieval step was performed by boiling slides in citrate buffer (10 μM citric acid, pH 6; Mallinckrodt, Hazelwood, MO) plus 0.05% Tween 20 (Sigma, St. Louis, MO) for 20 min and then incubated at RT for an additional 20 min. Slides were washed in 0.1% saponin (Sigma, St. Louis, MO)-PBS solution for 5 min. Endogenous peroxidases were quenched for 30 min by placing the slides in a 3% hydrogen peroxide (Fisher Scientific, Pittsburgh, PA) solution prepared with 0.1% saponin-PBS. Slides were washed for 5 min in 0.1% saponin-PBS before blocking for 30 min in 10% normal goat serum (KPL, Gaithersburg, MD). Rabbit anti-bovine Opn (generously supplied by Dr. Gary Killian; Pennsylvania State University, Almquist Research Centre, University Park, PA) was added to each slide (1:25, diluted in tris/PBS/BSA) and allowed to incubate overnight at 4 °C in a humidified chamber. A slide with normal rabbit serum served as a no primary antibody control for each tissue type. The following day, slides were washed in 0.1% saponin-PBS for 5 min and incubated at RT for 30 min with biotinylated goat anti-rabbit IgG (KPL, Gaithersburg, MD). Following a 5 min wash, slides were treated with streptavidin-horseradish peroxidase (KPL, Gaithersburg, MD) for 30 min. Slides were washed in 0.1% saponin-PBS and incubated in DAB-nickel substrate solution (Vector Laboratories, Burlingame, CA) for 20 min. Washed (3 × 5 min) slides were stained with Harris Hematoxylin (Newcomer Supplies, Middleton, WI). Slides were then washed (3 × 5 min) in water, treated with blueing water (ammonium hydroxide) for 1 min, and then washed an additional 3× (1 min/wash). Slides were then dehydrated by an ethanol series (water, 95%, 100%) and xylene, covered with Permount (Fisher Scientific, Pittsburgh, PA), and evaluated for the presence of Opn by light microscopy at 40× magnification.

2.3. Scoring of IHC

For evaluation of Opn in ileal tissue, 10 random fields of the lamina propria were scored for the frequency and intensity of cellular Opn immunoreactivity. For evaluation of the ICN, 10 random fields of the paracortex were scored. The frequency of cell staining was scored on a scale of 0–3 (0 = 0%, 1 = 25–75%, 2 = 25–75%, and 3 ≥ 75%). The intensity of the cell staining was also scored on a scale from 0–3 (0 = negative, 1 = mild intensity, 2 = moderate intensity, and 3 = intense).

2.4. Ziehl-Neelsen staining

Sections from the ileum and ICN were cut at 4–6 μm and stained with Ziehl-Neelsen (ZN) by conventional methods. Ten random fields of the lamina propria or the paracortex were scored for ileal tissue and IC node, respectively. Slides were scored on a scale of 0–3 (0 = no acid-fast bacilli, 1 = small number of bacilli, 2 = moderate number of bacilli, 3 = large number of bacilli). All slides were read at 40× magnification.

2.5. Statistical analysis

The data was analyzed using the PROC Mixed analysis of SAS (SAS, 2002). The model included infection group and frequency of cellular staining of intensity of staining. The values reported are means and standard errors with significance set at P < 0.05.

3. Results and discussion

We present here the first reported data on the localization of Opn in the ileum and ICN of dairy cows naturally infected with MAP. Infections caused by MAP, the causative agent of Johne’s disease (JD), are estimated to cost the US dairy industry upwards of $250 million annually (Ott et al., 1999). MAP is a weakly gram-positive, acid-fast bacillus. Calves most likely become infected during the first 6 months of life (Sweeney, 1996). Following initial infection, cows will remain in the subclinical, or asymptomatic, stage of the disease for several years until the disease progresses to the clinical state. The disease is associated with weight loss, diarrhea, and chronic granulomatous enteritis.

Osteopontin plays an early role in initiating the innate immune response to mycobacterial infections by promoting cellular adhesion and recruitment of inflammatory cells from the peripheral blood, increasing the number of activated macrophages, and eliciting a Th1 cytokine response. In the current study, macrophages, lymphocytes, and plasma cells in the ileal tissues of all animals stained positive for Opn (Table 1; Fig. 1A, C and E). This result is in contrast to that in the ICN, in which only lymphocytes stained positive for the control and subclinical groups (Table 1; Fig. 1B and D). Both lymphocytes and macrophages, however, stained positive for Opn in the ICN from clinically infected animals (Table 1; Fig. 1F). In all cell types, Opn stained within the cytoplasm. These results

<table>
<thead>
<tr>
<th>Infection group</th>
<th>Frequency of cellular staining</th>
<th>Staining intensity</th>
<th>Cell types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.43 ± 0.4a</td>
<td>1.93 ± 0.3</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Plasma cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Macrophages</td>
</tr>
<tr>
<td>Subclinical</td>
<td>2.25 ± 0.2ab</td>
<td>2.04 ± 0.3</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Plasma cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Macrophages</td>
</tr>
<tr>
<td>Clinical</td>
<td>2.93 ± 0.03b</td>
<td>1.57 ± 0.03</td>
<td>Macrophages</td>
</tr>
<tr>
<td>IC lymph node</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.42 ± 0.6</td>
<td>1.00 ± 0.5</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>Subclinical</td>
<td>2.25 ± 0.4</td>
<td>1.27 ± 0.3</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>Clinical</td>
<td>1.77 ± 0.4</td>
<td>0.64 ± 0.1</td>
<td>Lymphocytes/macrophages</td>
</tr>
</tbody>
</table>

Table 1
Frequency and staining intensity scores of osteopontin immunoreactivity in the ileum and ileocecal lymph node from dairy cows naturally infected with Mycobacterium avium subsp. paratuberculosis

Different superscripts indicate significant differences (P < 0.05).

are consistent with others who reported Opn secretion by activated macrophages and T-cells (Atkins et al., 1998; Ashkar et al., 2000). We have recently identified for the first time, Opn mRNA and protein expression in peripheral blood mononuclear cells isolated from healthy and MAP-infected dairy cows (Karcher et al., 2008). Osteopontin has also been identified in the testicular parenchyma of the bull reproductive tract (Erickson et al., 2007), and in cyclic bovine endometrium (Kimmins et al., 2004). Despite reports of Opn expression by enterocytes and smooth muscle cells, there was no staining of these cell types in either the ileum or ICN.

In the current study, clinical cows had poorly demarcated areas of severe granulomatous inflammation. These intestinal lesions were predominantly composed of macrophages immunoreactive for Opn (Fig. 1A). Diffusely, the lamina propria in clinical cows was expanded through invasion by an abundant number of macrophages. Additionally, the submucosa of
clinical cows was multifocally infiltrated and expanded by linear aggregates of macrophages. The staining of the macrophages appeared granular within the cytoplasm of the cell. Clinical cows had a greater percentage of cells staining positive for Opn compared with control cows ($P < 0.01$) (Table 1). Despite differences in the frequency of staining due to MAP infection, there were no differences observed in the intensity of the stained cells (Table 1). In contrast to the ileal sections, differences in the frequency of cells staining positive for Opn were not observed in the ICN between the different infection groups (Table 1). Progression of MAP infection to the clinical stage of the disease is characterized by increases in the granulomatous lesions in the lamina propria of the ileum. Macrophages become the predominant immune cell in the lesion and there is a dramatic increase in the bacterial load (Hostetter et al., 2005). The ability of Opn to recruit macrophages to sites of inflammation was illustrated in a study in which rats injected with recombinant Opn had a 225% increase in the number of macrophages at the injection site compared with the controls (Giachelli et al., 1998).

Staining by ZN method demonstrated acid-fast bacilli within the cytoplasm of macrophages in the lamina propria of the clinical Johne’s cows only (Table 2; Fig. 2A). Staining by ZN method demonstrated acid-fast bacilli in the paracortex of the ICN for clinical cows only (Table 2; Fig. 2B). Control and subclinical cows were negative for acid-fast bacilli (data not shown).

The importance of Opn in controlling mycobacterial infections was established when Opn knock-out mice were challenged with $M. \text{bovis}$ Bacillus Calmette-Guerin (BCG Pasteur). These mice suffered from more severe infection, heavier bacterial loads, and greater granuloma burdens compared to the wild-type mice (Nau et al., 1999). An inverse correlation has been noted between the amount of Opn in tissues and disease severity in patients with mycobacterial infections (Nau et al., 1999). In this study, the presence of Opn was positively correlated with granulomatous lesions in the ileum of clinically infected cows. As JD progresses to the clinical stage of the disease, the intestinal mucosa becomes corrugated and thickened (Clark, 1997) and there is a transition from the cell-mediated to humoral

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Table 2

<table>
<thead>
<tr>
<th>Infection group</th>
<th>Score $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ileum</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0$^a$</td>
</tr>
<tr>
<td>Subclinical</td>
<td>0$^a$</td>
</tr>
<tr>
<td>Clinical</td>
<td>2.73 ± 0.2$^b$</td>
</tr>
<tr>
<td><strong>IC lymph node</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0$^a$</td>
</tr>
<tr>
<td>Subclinical</td>
<td>0$^a$</td>
</tr>
<tr>
<td>Clinical</td>
<td>1.97 ± 0.6$^b$</td>
</tr>
</tbody>
</table>

$^a,b$Different superscripts indicate significant differences ($P < 0.05$).

$^a$ 10 random fields of lamina propria scored.

$^b$ 10 random fields of paracortex scored.

$^c$ Score: 0 = no acid-fast bacilli, 1 = small number, 2 = moderate number, 3 = large number.

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Fig. 2. Representative section of ileal (A) and ileocecal lymph node (B) tissues obtained post-mortem from a dairy cow clinically infected with *Mycobacterium avium* subsp. *paratuberculosis*. Sections were stained for acid-fast bacilli using the Ziehl-Neelsen method. Acid-fast bacteria were localized within the cytoplasm of macrophages in the lamina propria (A) and in the paracortex (B). ($\times 40$) Bar, 50 $\mu$m.
immune response. This transition has been documented by the upregulation of IL-10 and TGF-β in ileal tissues from clinical JD cows (Khalifeh and Stabel, 2004). However, B-cells from clinical JD cows fail to proliferate in response to antigen stimulation (Waters et al., 1999), providing little protection to the persisting infection. Interestingly, mice deficient in Opn expression had increased IL-10 production and decreased macrophage function (Ashkar et al., 2000). The increase in Opn in the ileum of clinically infected cows may be a last minute effort by the host immune response to elicit a protective cell-mediated immune response. Recent work in our laboratory demonstrated greater expression of Opn mRNA in nonstimulated PBMC isolated from dairy cows with subclinical paratuberculosis compared with noninfected healthy cows and clinically infected animals in the periparturient period (Karcher et al., 2008). In contrast, upon stimulation of PBMC with a whole-cell sonicate of MAP, higher Opn expression was noted in cells from control and clinical cows (Karcher et al., 2008). Interestingly, osteopontin expression correlated positively with the expression of other Th1 cytokines, IFN-γ, IL-1α, and IL-12 in PBMC isolated from clinically infected cows. Collectively, these results suggest that a systemic upregulation of Opn may be occurring after initial exposure to MAP and in the early stages of infection, followed by a localized upregulation in the target tissue(s) in the more clinical stages, with both processes calculated to mediate MAP infection (Karcher et al., 2008).

In conclusion, the results of this study demonstrate for the first time the presence of Opn in the ileal and ICN tissue sections from healthy dairy cows and cows naturally infected with MAP. This study also established that Opn is upregulated in areas of granulomatous inflammation, which is consistent with those of previous reports in other species.

References


