Short Communication

Osteopontin Expression Correlates with Clinical Outcome in Patients with Mycobacterial Infection

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Osteopontin (OPN) is a protein that is expressed in chronic inflammatory diseases including tuberculosis, and its deficiency predisposes to more severe mycobacterial infections in mice. However, no reports have identified altered OPN expression in, or correlated these alterations to, infections in humans. The data presented herein identify alterations in the tissue expression of OPN protein and describe an inverse correlation between these levels and disease progression after inoculation of *Mycobacterium bovis* bacillus Calmette-Guérin vaccine in humans. Patients with regional adenitis and good clinical outcomes had abundant OPN in infected lymph nodes. This pattern of OPN accumulation was also observed in patients infected by *M. avium-intracellulare*. In contrast, patients with disseminated infection and histologically ill-defined granulomas had no significant osteopontin accumulation in infected lymph nodes; these patients had either deficiencies in the interferon- γ receptor 1 or idiopathic immune defects. The level of OPN protein expression was inversely correlated with disseminated infection and, of particular interest, with death of the patient. We conclude that osteopontin expression correlates with an effective immune and inflammatory response when humans are challenged by a mycobacterial infection and that osteopontin contributes to human resistance against mycobacteria. (Am J Pathol 2000, 157:37–42)

The clinical spectrum of tuberculosis is changing with alarming trends toward antibiotic resistance throughout the world¹ and the emergence of new, virulent strains.² Immunization with an attenuated mycobacterium, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), has been widely used to minimize the clinical impact of *M. tuberculosis* (MTB). However, this live vaccine can cause serious infection in some recipients, many of whom do not have recognizable immunodeficiencies.^{3,4} Recently, multiple reports have identified genetic abnormalities in patients with increased susceptibility to mycobacterial infection.^{5–10} These patients are characterized by disseminated disease from bacteria with low virulence, such as BCG. In some patients, the absence of the high-affinity interferon- γ receptor (IFN γ R1) leads to significant vulnerability to mycobacterial infections, which are often fatal.^{8,10} Dominant negative mutations of IFN γ R1 also increase susceptibility to mycobacterial infections, but most of these patients survive their infection.⁹ Finally, partial recessive IFN γ R1 deficiency, IFN γ R2 deficiency, and interleukin-12R or interleukin-12 deficiencies predispose to significant, but treatable, mycobacterial infections.^{5–7,11,12}

We have studied the interaction between macrophages and mycobacteria to gain insight into host defense mechanisms and, ultimately, to identify possible diagnostic and therapeutic targets. Using a system of cDNA library screening of macrophages infected with BCG, we discovered an association between osteopontin (OPN) and infections with mycobacteria.¹³ OPN is a secreted protein that is expressed in chronic inflammatory diseases, particularly tuberculosis.^{13,14} It is produced by macrophages, T cells, and natural killer cells on activation.¹⁵ The protein is known to activate adhesion and migration of several cell types,^{15,16} including macrophages.¹⁷ OPN also affects T-cell migration, adhesion, and proliferation *in vitro*.¹⁸

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Experiments using OPN null mice support the idea that this protein enhances host resistance to infection. Mice that lack OPN are more susceptible to mycobacterial infection with a delayed clearance of the mycobacteria.¹⁹ These animals are also more susceptible to tumor progression and have impaired wound remodeling, likely because of reduced macrophage migration and activity.^{20,21} Thus, OPN seems to modulate macrophage function and its absence impairs host responses in mice.

OPN has also been detected in several pathological conditions.^{13,14,22–25} The amount of tissue OPN expression in glioblastomas has been directly correlated with the tumor's histological grade.²⁶ Serum levels of OPN are directly correlated with the burden of metastatic breast cancer and are inversely correlated with survival.²⁷ However, there have been no reports of altered levels of OPN expression as an indicator of the host response during infection. The objective of the current study was to determine whether OPN expression varied according to the severity of mycobacterial infections. We tested for OPN expression in tissue from patients suffering from infection after inoculation with BCG vaccine, both from patients with localized infection and from patients with disseminated infection. The results demonstrate that OPN expression varied between patients with infection and that the level of expression correlated with the clinical outcome for the patient.

Materials and Methods

Patient Specimens

The clinical features and genetic defects of the patients with severe BCG or nontuberculous mycobacterial infection have been reported elsewhere. These patients have been found to have recessive complete IFN γ R1 defects (C2), recessive partial IFN γ R1 defects (P1), and dominant negative IFN γ R1 defects (DN1, DN2).^{8,9,11} One patient (C1) with disseminated disease is included in another manuscript.³⁸ Two other patients with severe infection have uncharacterized immune defects (ID1, ID2).^{3,28} Several patients with localized, benign infection by BCG or nontuberculous mycobacteria and no identifiable underlying immunodeficiency are included in this report: L1 is a female who presented at 3 months, L2 is a female who presented at 6 months, L3 is a female who presented at 2.6 years, and L4 is a male who presented at 2.1 years.

Immunohistochemistry

Specimens were processed with standard histological techniques. Paraffin-embedded sections were dewaxed with HistoClear (National Diagnostics, Atlanta, GA) and rehydrated with graded alcohol. The sections were treated with pronase for 6 minutes (DAKO Corporation, Carpinteria, CA) followed by 0.01% azide/0.3% hydrogen peroxide in water for 20 minutes to inactivate endogenous peroxidases. After blocking with 20% goat serum in phosphate-buffered saline (PBS), primary antibodies (1% in 10% goat serum/PBS) were incubated for 2 hours at

room temperature. Antibodies MPIIB10, specific for osteopontin, and QH-1, an isotype control specific for quail endothelium, were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Antibody KP-1, specific for human CD68 on macrophages, was obtained from DAKO. The secondary goat anti-mouse immunoglobulin G (5 μ g/ml), the avidin-biotin tertiary reagents, and the color substrates were from Vector Laboratories (Burlingame, CA). After counterstaining with hematoxylin (DAKO) or methyl green (Vector), samples were dehydrated in graded ethanol and Clear-Rite 3 (Richard-Allan Scientific, Kalamazoo, MI) before mounting with Permount (Fisher, Fair Lawn, NJ).

OPN Scoring and Statistical Analysis

A simple histological score was devised to estimate the amount of OPN present in tissue sections. Macrophages and giant cells, identified by morphology and by CD68 staining, and lymphocytes, identified by morphology, were individually scored for OPN with the following scale: >50% of cells positive = 2, <50% of cells positive = 1, no cells positive = 0.

Statistical analyses were performed using Statview software (SAS Institute, Inc., Cary, NC). Two-tailed Student's *t*-test was used to compare mean $\pm$ SD of OPN scores. Contingency tables and chi-square analysis of OPN score versus dissemination or clinical outcome were set up with an OPN score cutoff of 2. *P* values <0.05 were considered statistically significant.

Results

Osteopontin Expression in Patients with Localized Infection

OPN is closely associated with granulomatous inflammation in humans infected by MTB.^{13,14} Before analyzing patients with disseminated BCG infection, it was important to determine whether OPN is normally expressed in humans infected by mycobacteria other than MTB. To investigate this question, pathological lymph nodes obtained from patients with localized infection after inoculation with BCG vaccine or with localized *M. avium-intracellulare* infection were stained for OPN protein. The tissues of these patients showed histological features characteristic of a mycobacterial infection: well-formed granulomas of histiocytes surrounded by lymphocytes and the formation of giant cells. As shown in Figure 1A, localized adenitis after BCG inoculation resulted in extensive OPN accumulation identified by the MPIIB10 monoclonal antibody (Figure 1A). Although *M. avium-intracellulare* is not a member of the MTB complex, this organism also elicited OPN accumulation in lymph node granulomas of a host with localized disease (Figure 1B). The presence of macrophages was confirmed on adjacent sections that were stained for CD68 (Figure 1, C and D). Thus, OPN expression in BCG or *M. avium-intracellulare* infection co-localized with macrophages and giant cells, similar to what was observed with pulmonary MTB infec-

Figure 1. OPN expression in normal patients with localized adenitis. OPN protein detected in tissue sections by immunohistochemistry with monoclonal antibody MPIIB10 and diaminobenzidine substrate. **A and B:** Widespread expression of OPN was identified with the brown color of the diaminobenzidine in numerous cell types including histiocytes and lymphoid cells with darkly staining nuclei identified by hematoxylin. **C and D:** The presence of macrophage and giant cells was confirmed with staining for CD68, red color from Vector red substrate and methyl green counterstain. Isotype control antibody for MPIIB10 showed no (**E**) or little (**F**) background staining (diaminobenzidine substrate, hematoxylin counterstain). Original magnification, $\times 100$.

tion.¹³ In addition, similar widespread staining of lymphoid cells has been observed in pulmonary tuberculosis infections.¹³ An isotype control antibody for MPIIB10 failed to show significant staining (Figure 1, E and F).

Osteopontin Expression in Patients with Disseminated Infection

Having established the pattern of OPN expression in patients with localized adenitis, we tested samples from patients with disseminated BCG infection.

Recessive Complete IFN γ RI Deficiency

Patients with a complete deficiency of IFN γ RI showed no significant OPN accumulation in infected lymph nodes

(Figure 2, A and C) despite numerous macrophages identified by staining for CD68 (Figure 2, B and D). These patients succumbed to their infections despite antimicrobial therapy.

Recessive Partial IFN γ RI Deficiency

Tissue from one patient with a recessive, partial defect in IFN γ RI expression demonstrated less intense OPN staining (Figure 2E) compared to those with localized disease (Figure 1, A and B), but more than that seen in patients with a complete deficiency (Figure 2, A and C). The OPN expression co-localized with macrophages seen on an adjacent section (Figure 2F). This same patient had a milder clinical course and survived the disseminated BCG infection after antibiotic therapy.

Figure 2. OPN expression in patients with immune defects. Sections were stained for OPN (brown, diaminobenzidine substrate) and CD68 (Vector red substrate). Patients with complete deficiency of IFN γ RI showed no significant OPN accumulation (**A** and **C**) in macrophages within the section (**B** and **D**). A patient with partial deficiency of IFN γ RI demonstrated OPN (**E**) present in macrophages (**F**). A patient with a dominant negative IFN γ RI had no OPN detectable (**G**) although macrophages were present (**H**). Two patients with idiopathic immune defects had no (**I**) or little (**K**) detectable OPN although macrophages were identified (**J** and **L**). In some specimens there were occasional OPN-positive cells with small, dark nuclei interpreted as lymphoid cells (**C** and **K**). There was no significant staining with control antibody in these specimens. Original magnification, $\times 100$.

Dominant Negative IFN γ RI

In contrast to the patient with the recessive partial receptor defect, two patients with dominant negative mutations in IFN γ RI demonstrated patterns of OPN expression more like those patients with complete receptor deficiencies. Tissue from one of these patients is shown in Figure 2G. The specimen showed no OPN in the infected lymph node although macrophages were present (Figure 2H).

Idiopathic Immune Defect

Finally, tissue from two patients with idiopathic immune defects were tested (Figure 2, I–L). These patients also had no or little OPN demonstrated in infected lymph nodes (Figure 2, I and K) despite macrophage infiltration (Figure 2, J and L). In some instances, patients with immune defects demonstrated low levels of staining in cells with a lymphocyte morphology; see Figure 2, C and K. This pattern of staining appeared distinct from the macrophage staining seen in other samples and may represent direct cellular activation, such as by a T-cell antigen receptor which can induce OPN production.²⁹

A summary of the patient samples analyzed in this study is listed in Table 1. Patients with local disease tended to have high levels of OPN expression. Conversely, patients with immune defects tended to have

absent or low levels of OPN expression. Statistical analyses verified correlations between OPN expression and either dissemination or clinical outcome. The mean OPN scores (mean $\pm$ SD) were statistically different by Student's *t*-test in both analyses for dissemination: localized (3.75 ± 0.5) versus disseminated (0.71 ± 0.76), $P = 0.0065$; and for outcome: alive (3.00 ± 1.26) versus dead (0.40 ± 0.55), $P = 0.0086$. On contingency tables, an OPN score of <2 was associated with dissemination whereas a score of ≥ 2 was associated with localized disease (chi-square = 7.54, $P = 0.0060$). Similarly, an OPN score of <2 was associated with death whereas a score of ≥ 2 was associated with survival after infection (chi-square = 7.64, $P = 0.0057$).

Discussion

This study is the first to identify variations in the tissue levels of OPN as an indicator of the host response to infection and to demonstrate that these variations correlate with the severity of the infection. We have analyzed clinical specimens to explore immunodeficiency and increased susceptibility to infection by poorly virulent mycobacteria. By characterizing OPN expression in tissues from patients with localized and disseminated disease, we have identified an inverse correlation between levels of OPN in tissues and disease severity. In general, pa-

Table 1. Summary of Cases in This Report

Patient	Organism	Location	OPN-Mac	OPN-Lymph	Total Score	Outcome	Reference
L1*	BCG	Local	2	2	4	Alive	This report
L2	BCG	Local	1	2	3	Alive	This report
L3*	MAI	Local	2	2	4	Alive	This report
L4	MAI	Local	2	2	4	Alive	This report
C1*	BCG	Diss	0	0	0	Dead	38
C2	BCG	Diss	0	1	1	Dead	8
DN1*	BCG	Diss	0	0	0	Dead	9
DN2	BCG	Diss	0	1	1	Alive	9
P1*	BCG	Diss	2	NS [†]	2	Alive	11
ID1*	BCG	Diss	0	1	1	Dead	3
ID2*	BCG	Diss	0	0	0	Dead	28

L, localized; C, complete IFN γ RI deficiency; DN, dominant negative IFN γ RI deficiency; P, recessive partial IFN γ RI deficiency; ID, idiopathic deficiency; Mac, macrophage; Lymph, lymphocyte; Diss, disseminated.

*Illustrated in Figures 1 and 2.

[†]Not scored, cannot clearly discern lymphocyte morphology.

tients who do well after an infection by mycobacteria express high levels of OPN.

The current findings further substantiate the beneficial effects of OPN accumulation during infection. Several reports indicated that allelic variation of the Ric locus, now known to be OPN,³⁰ affected host susceptibility to infection.^{31–33} BCG infection of OPN null mice demonstrated the absence of OPN led to more severe infections.¹⁹ This indicated the protein has a salutary effect on the host's efforts to eradicate the BCG. Recent data have shown that OPN can activate interleukin-12 production from macrophages and can enhance Th1-type cell-mediated immunity.³⁴ The data from human tissues infected with mycobacteria show a correlation between OPN expression and clinical outcome: patients did better when there was OPN in abundance after infection. Taken together, these observations support the idea that OPN enhances host resistance to mycobacterial infections in both mice and humans. Based on mouse studies, the effects of OPN are likely to involve the activation of macrophages and the initiation of a Th1-type response.

In the patient samples studied, the expression of OPN by macrophages was closely linked to functional interferon- γ (IFN- γ) receptors, suggesting that IFN- γ signaling is important for accumulation of OPN *in vivo*. Consistent with this inference is the fact that the promoter region of the OPN gene has an IRF-1 binding sequence.³⁵ IFN- γ and its receptor are key components of the host response against MTB.^{36,37} It is not known if the two patients with idiopathic immunodeficiency and disseminated BCG infection had defects with the pathway of IFN- γ signaling. However, it is possible that there is a primary defect in OPN production by macrophages or another defect that secondarily reduces OPN production in some patients with immune deficiency. Based on animal studies, one would predict that a primary defect in OPN production would lead to greater bacterial burden and delayed resolution of infection, leading to death in some instances.^{19,32} In any case, increases of OPN expression may represent a common endpoint of several pathways of macrophage activation.

Because tissue expression of OPN was associated with a successful outcome after infection, it may be possible to use OPN as a tool to measure the competency of

host defenses. OPN may be of diagnostic value to identify patients with severe underlying immunodeficiencies such as the IFN- γ signaling defects or the idiopathic immune defects tested here. In addition, measuring OPN responses *in vitro* before administering a vaccine, such as BCG, may be useful to test vaccine safety in a particular host. Prospective studies will be required to determine whether OPN expression is useful to predict the integrity of host responses against infection.

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