Potential Roles of Follicular Dendritic Cell–Associated Osteopontin in Lymphoid Follicle Pathology and Repair and in B Cell Regulation in HIV-1 and SIV Infection

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Osteopontin is a secreted, phosphorylated, acidic glycoprotein that was first identified in studies of bone repair but was subsequently shown to have multiple functions in the repair of other tissues, immune responses, and inflammation [1, 2]. In mineralized tissues, osteopontin binds to the extracellular matrix through interactions between its asparagine-glycine-aspartate sequence and integrins, and it regulates calcium deposition during the stress-induced remodeling of bone. In other tissues, osteopontin promotes wound healing and inhibits ectopic calcification [3–5]. Osteopontin also functions in the induction of cellular and humoral immune responses [6–8] and participates in both physiological inflammation during wound healing and immune responses and pathological inflammatory conditions that include arthritides [9, 10], multiple sclerosis [11], and autoimmune diseases [8, 12, 13].

We recently found that osteopontin gene expression increased in HIV-1–infected lymphatic tissues after treatment, and we undertook mapping experiments to study osteopontin’s possible functions in this context. We discovered species-specific colocalization of osteopontin with the follicular dendritic cell (FDC) network in lymphatic tissues in HIV-1 and simian immunodeficiency virus infections, and we found that changes in FDC-associated osteopontin covary with changes in lymphoid follicles during acute and late stages of infection and in response to treatment. We propose that this localization normally facilitates antibody production and plays a role in B cell abnormalities in infection and in the reconstitution of lymphoid follicles with treatment and that mapping genes identified in microarray studies is a useful experimental approach to gaining a better understanding of function in the context of a particular tissue and disease.
production. In support of this hypothesis, we document (1) changes in FDC-associated osteopontin that parallel the well-known hypergammaglobulinemia and B cell abnormalities observed during the early stages of HIV-1 and simian immunodeficiency virus (SIV) infections, (2) decreases in FDC-associated osteopontin that parallel the destruction of lymphoid follicles during late stages of infection, and (3) during HAART, a coordinate normalization of follicular architecture and FDC-associated osteopontin.

Material and Methods

**Human lymph-node biopsies.** After we obtained signed, informed consent, inguinal lymph-node biopsies were performed, by use of standard surgical techniques, on individuals under local anesthesia who were participating in University of Minnesota institutional review board-approved protocols [15]. A portion of the tissue was placed in 4% paraformaldehyde for 3–4 h and was then transferred to 70% ethanol and embedded in paraffin for in situ hybridization, immunohistochemical staining, and histological examination.

**Lymph-node biopsies from SIV-infected rhesus macaques.** Axillary lymph-node biopsies were performed on adult female SIV- and simian retrovirus–negative rhesus macaques under anesthesia before and 1 and 4 weeks after intravenous (iv) inoculation with 2 × 10⁴ ID₅₀ of SIVmac239 (provided by R. Desrosiers, New England National Primate Research Center, Southborough, MA). All animal housing, care, and research were performed in accordance with the Guide for the Care and Use of Laboratory Animals [16] and with protocols approved by the Institutional Animal Care and Use Committee of the National Cancer Institute.

**Immunohistochemical staining.** For immunohistochemical staining, after blocking to reduce nonspecific binding, sections were reacted sequentially with primary antibody, biotinylated secondary antibody, and ABC reagent, and then were stained with diaminobenzidine and counterstained with hematoxylin/eosin. For osteopontin staining, we used monoclonal antibodies to human osteopontin peptides at the C terminus 3 aa from the thrombin cleavage site (Immuno-Biological Laboratories), goat polyclonal antibodies to full-length recombinant mouse osteopontin (AF808; R&D Systems) and proteolytically derived N- and C-terminal fragments (provided by L. Fisher, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD) [17, 18], rabbit polyclonal antibodies to full-length and N- and C-terminal fragments of recombinant mouse osteopontin (provided by L. Liaw, Maine Medical Center Research Institute, Scarborough, ME), and rabbit polyclonal antibodies that recognize full-length and C-terminal fragments of osteopontin and cross-react with human, pig, and dog osteopontin (abcam 8448; Novus Biologicals).

**In situ hybridization.** Tissue sections of 8 µm were cut and adhered to slides, deparaffinized, and subsequently pretreated for the detection of HIV RNA by in situ hybridization, as described elsewhere [19]. In brief, sections were pretreated with HCl, digitonin, and proteinase K, to enhance the diffusion of probes, and then acetylated, to reduce the nonspecific binding of probes. After the hybridization of a collection of ³⁵S-labeled HIV-1–specific riboprobes, sections were washed, treated with ribonuclease, dehydrated, coated with NTB2 (Kodak), and developed and stained after radioautographic exposure. For osteopontin, cDNA from the American Type Culture Collection (ATCC number 61052) was subcloned into the pBluescript II SK(−) plasmid, and an ³⁵S-labeled riboprobe was prepared by incorporation of the label with T7 RNA polymerase.

**Statistical analysis.** A random-effects model was used to determine the statistical significance of changes in the percentage of FDC-associated osteopontin/lymphatic tissue area attributable to HAART. This analysis was necessary for combining information from 2 small, slightly different studies. The model assumed that the changes in each individual were normally distributed, given some study-specific mean effect, and that these study-specific mean effects were drawn from another normal distribution. The mean of the latter distribution was the estimate of the effect due to HAART. A Bayesian approach was used, and computation was done by first integrating the variance parameters out of the posterior distribution, then directly sampling the other 3 parameters from a histogram approximation of their posterior distribution. Calculations were conducted by use of S-plus software (version 3.4; MathSoft).

Results

**FDC-associated osteopontin in HIV-1–infected and –uninfected lymphatic tissues as revealed by mapping studies.** With the rationale that knowing where osteopontin was expressed in lymphatic tissues would provide additional clues to its function, we determined sites of osteopontin expression by immunohistochemical staining. We had expected from the results of other studies [1, 20] that we would detect osteopontin in cells, but we had not seen a report of an association of osteopontin with lymphoid follicles. We were thus surprised to find that (1) before treatment, during the late stage of HIV-1 infection, osteopontin colocalized with the FDC network in small residual secondary follicles; and (2) 2 months after the initiation of HAART, there was increased osteopontin staining in a pattern characteristic of the FDC network in the large follicles that had reformed by that time (Figure 1A and 1B). We confirmed the colocalization of osteopontin protein with the FDC network by staining for the FDC marker CD35 and by revealing, through in situ hybridization, the virions bound to the FDC network [19] before treatment (Figure 1C and 1D).
Figure 1. Follicular dendritic cell (FDC)-associated osteopontin.

A and B, Detection of FDC-associated osteopontin by immunohistochemical staining with a monoclonal antibody to a human osteopontin peptide at the C terminus in an HIV-1–infected individual in the late stages of infection before (A) and after (B) 2 months of highly active antiretroviral therapy (HAART). Note the follicular pattern of staining and increased size of the follicle and osteopontin staining after HAART.

C and D, Double-label in situ hybridization to detect HIV-1 RNA in virions bound to the FDC network and immunohistochemical staining for osteopontin.

C, Bright silver grains after in situ hybridization with 35S-labeled HIV-1–specific riboprobes to detect FDC-associated virions colocalizing with brown-staining osteopontin.

D, Double-label for CD35 to mark the FDC network (red stain) colocalizing with gray-staining osteopontin showing colocalization.

E, Osteopontin staining at high magnification (×40) of the FDC network in an HIV-1–uninfected individual.
We then investigated and documented osteopontin colocalization with the FDC network in follicles in other HIV-1–infected and –uninfected individuals (Figure 1E).

**Binding but not production of osteopontin by FDCs.** Although the FDC network clearly binds osteopontin, we did not detect osteopontin mRNA in FDCs by in situ hybridization, although we did detect osteopontin mRNA (Figure 2A) in cells in the paracortical T cell zone identified by double-label staining as T cells and macrophages (Figure 2B and 2C). We conclude that these cells are the local source of osteopontin that binds to the FDC network.

**Increase in FDC-associated osteopontin during HAART.** We found that the size of the follicles and FDC-associated osteopontin gene expression (Figure 1A and 1B) increased in parallel, which suggests that follicle formation and osteopontin synthesis and binding are concurrently regulated. Because the FDC network is slowly reconstituted during HAART [21], we would therefore expect corresponding increases in FDC-associated osteopontin gene expression. We quantified FDC-associated osteopontin as a percentage of the area of lymphatic tissues stained by antibody in a total of 12 individuals in 2 separate studies over the course of 1.5 years of HAART; indeed, we found, in a meta-analysis of the separate studies, statistically significant increases ($P = .02$) in FDC-associated osteopontin gene expression that closely matched the increases previously documented in the FDC network (data not shown) [21].

**Increase in FDC-associated osteopontin gene expression during acute SIV infection.** The increases in both osteopontin gene expression and the number and size of lymphoid follicles imply that FDC-bound osteopontin gene expression should also increase with the well-documented and particularly dramatic increases in the numbers and sizes of the follicles during the early stage of HIV-1 infection [22]. For obvious practical and ethical reasons, lymph-node biopsies cannot be obtained during the relevant time frame (before and shortly after HIV-1 infection) for testing this hypothesis. However, we were able to study lymph nodes obtained before and during acute SIV infection of rhesus macaques, a nonhuman primate model in which the changes in lymphatic tissues correspond closely to those in HIV-1.
Figure 3. Osteopontin in follicles and cells of a simian immunodeficiency virus (SIV)-infected rhesus macaque.

A, Follicular dendritic cell-associated osteopontin and 2 cells (arrow, inset).

B, Increased osteopontin in follicles in early SIV infection. Increased osteopontin staining, using the monoclonal antibody to the human osteopontin peptide described in the Figure 1 legend, paralleled the increased nos. and sizes of follicles in biopsies from 3 macaques from baseline to 1 and 4 weeks after intravenous infection.
infection [23, 24]. Osteopontin colocalized to the rhesus macaque FDC network (Figure 3A) and to cells in the paracortex (Figure 3A, inset); in accordance with the hypothesis, FDC-bound osteopontin gene expression increased substantially and proportionately with the increased numbers and sizes of the follicles accompanying immune activation in response to infection at 1 and 4 weeks after IV infection with SIV (Figure 3).

**No detection of FDC-associated osteopontin gene expression in the mouse.** In addition to the follicular hyperplasia described above, polyclonal B cell activation and hypergammaglobulinemia are also characteristic of early HIV-1 infection [24–27]. Because the increased FDC-associated osteopontin gene expression observed in acute SIV infection pointed to a potential role of osteopontin in these B cell abnormalities that we could not directly test in fixed tissues, we designed experiments with model antigens to examine the relationship between osteopontin gene expression and antibody production in mice. Because we had not seen descriptions of osteopontin colocalizing with the FDC network in mice, and because of the many examples of differences in mouse and human immune systems [28], we first undertook some preliminary experiments to determine whether osteopontin was, in fact, associated with murine FDCs. Although the osteopontin antibodies readily detected osteopontin-positive cells in the paracortex and follicles (Figure 4) of mouse (B6 and other strains) lymph nodes, there was no detectable FDC-associated osteopontin in polyclonal and monoclonal antibodies to full-length recombinant mouse osteopontin and proteolytically derived N- and C-terminal fragments. Thus, osteopontin’s association with the FDC network is species specific.

**Discussion**

In the present studies, we found that (1) there is a species-specific association of osteopontin with the FDC network and (2) changes in the levels of FDC-associated osteopontin gene expression are correlated with pathological abnormalities in lymphoid follicles in HIV-1 and SIV.
infections and the reparative response to HAART. We now discuss what these findings might imply about FDC-associated osteopontin’s roles, both normally in antibody production and in immunodeficiency virus infections.

The association of osteopontin with the FDC network locates a powerful antibody-stimulating cytokine in the lymphoid follicle structure, which is organized to bring together antigen bound to the FDC network, B cells, and germinal-center CD4+ cells to stimulate B cell proliferation and differentiation for the production of high-affinity antibodies. The FDC-associated osteopontin could be contributing to antibody production at this site by facilitating FDC–CD4+ cell germinal-center interactions that augment Th1 cytokine and CD40L expression. Because osteopontin is known to augment CD40L expression in T cells [32], FDC-associated osteopontin is ideally situated anatomically to increase the expression of CD40L on CD4+ cells in close proximity to B cells, thereby inducing B cell proliferation and antibody production.

Osteopontin in mice, similarly, has long been known to stimulate antibody production and B cell proliferation through CD40-CD40L and other interactions with CD4+ cells. T cells and macrophages also produce osteopontin in mice, just as we have shown in humans and nonhuman primates. However, osteopontin was not detectably bound by FDCs in mice, in contrast to what was seen in primate species. Although the murine and human osteopontin sequences are closely related [33–35], we conclude from the present studies that the additional sequences that osteopontin has acquired through evolution enable it to bind to FDCs for the facilitation of antibody production.

In all 3 species, elevated levels of osteopontin were implicated in B cell abnormalities. In mice, high levels of osteopontin were associated with polyclonal B cell activation, autoimmunity disease, and murine AIDS, respectively, in MRL/lpr mice, osteopontintransgenic mice, and mice infected with the retrovirus LP-BM5 [8, 12, 13, 20]. In human and nonhuman primate lymphatic tissues, changes in the levels of FDC-associated osteopontin that could be contributing to the B cell abnormalities observed in HIV-1 and SIV infections and to the pathological changes in lymphoid follicles in these infections that cover a spectrum from follicular hyperplasia, to involution, to the development of lymphomas [22, 29]. During the early stages of infection, the deposition of viral immune complexes in the FDC network is associated with increases in the number and size of the follicles and with the concomitant generalized polyclonal activation of B cells that results in hypergammaglobulinemia [24, 26, 27, 30]. Recent evidence has suggested that hyperactivated naive B cells are responsible for increased immunoglobulin production [31] and that the abnormal differentiation of naive B cells is driven by CD40-CD40L interactions that would be enhanced by FDC-associated osteopontin gene expression.

By the later stages of infection, HIV-1 and SIV have destroyed lymphoid follicles, with accompanying defects in the humoral immune response to recall antigens and vaccination that reflect structural damage and reductions in the number of memory B cells [31]. We had shown previously that this destructive process is reversible by HAART. During HAART, follicles reformed over a period from a few months to 1.5 years of treatment; by the latter time point, levels were similar in proportion to numbers of follicles in HIV-1–uninfected individuals [36]. Here, we have provided encouraging evidence that the capacity to regenerate FDC-associated osteopontin is retained as another component of the immune reconstitution that takes place during HAART.

Finally, the present studies illustrate how mapping the cellular and anatomic location of genes discovered in microarray studies can help define function in a particular tissue and disease. In particular, in the immunodeficiency virus infection of lymphatic tissues, there would have been no a priori reason or literature on osteopontin to suspect binding to FDCs and, therefore, a potentially new role in normal and abnormal B cell states. Thus, mapping studies can complement gene profiling by pointing the way forward from hypothesis generation to hypothesis testing.

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