Acceleration and Increased Severity of Collagen-Induced Arthritis in P-Selectin Mutant Mice


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Acceleration and Increased Severity of Collagen-Induced Arthritis in P-Selectin Mutant Mice


P-selectin plays an important role in leukocyte adherence to microvascular endothelium and is expressed in synovial tissue from patients with rheumatoid arthritis (RA). However, the contribution of P-selectin to the initiation and chronicity of joint inflammation is not well understood. In these studies, collagen-induced arthritis (CIA) was induced in P-selectin mutant (−/−) mice to explore the role of P-selectin in the development of joint inflammation. Surprisingly, CIA onset was accelerated and severity was increased in P-selectin mutant mice, compared with wild-type mice (+/+). Increased levels of anti-type II collagen IgG were detected in both nonarthritic and arthritic P-selectin mutant mice from days 14–91. In addition, splenocytes isolated from immunized and nonimmunized P-selectin mutant mice produced significantly less IL-2 and IL-4, but significantly higher levels of IL-10 and IL-5 than splenocytes from wild-type mice. These observations show that P-selectin-mediated leukocyte rolling is not required for the development of murine CIA and that P-selectin expression exerts a controlling effect on the development of Ag-driven inflammatory joint disease, possibly by mediating the recruitment and/or trafficking of specific leukocyte subtypes into lymphoid tissue or inflammatory foci. The Journal of Immunology, 1999, 163: 2844–2849.

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uukocyte rolling on the endothelial cell surface is the first step in the emigration of leukocytes from the vasculature into tissue during an inflammatory response (reviewed in Ref. 1). This process is mediated in part by the selectins (P-, E-, and L-selectin), a group of proteins that share a common structure, including an N-terminal calcium-dependent lectin binding domain (reviewed in Refs. 2 and 3). The selectins bind to several different carbohydrate structures, and many of the selectin ligands are mucins. P-selectin is expressed on endothelial cells, megakaryocytes, and activated platelets. Expression of E-selectin is limited to endothelial cells, and up-regulation requires gene transcription stimulated by mediators such as IL-1 and TNF-α. L-selectin is expressed on the majority of leukocytes and is shed from the cell surface following activation.

The contribution of P-selectin to leukocyte rolling and emigration has been well documented. Inhibition or loss of P-selectin results in a significant reduction of neutrophil rolling and acute emigration in many inflammatory models (4–9). In addition, in vitro flow chamber studies have shown that P-selectin also participates in both lymphocyte and monocyte rolling on endothelial cells (10–13). The functional role of P-selectin in mediating leukocyte recruitment during chronic inflammatory diseases has only recently been investigated (14–18). Several studies provide evidence that P-selectin expression is important in the development of joint inflammation during rheumatoid arthritis (RA) (19–22). P-selectin is expressed on both cultured synovial microvascular endothelial cells and on synovial tissue endothelium, and increased levels of soluble P-selectin have been reported in synovial fluid from RA patients (23–26). Also, Grober et al. (27) showed that monocyte adherence to microvascular endothelium in RA synovial tissue can be blocked with anti-P-selectin Abs using the Stamper-Woodruff assay, suggesting that P-selectin may promote monocyte rolling and emigration from the synovial vasculature.

Murine collagen-induced arthritis (CIA) is a well-known model of chronic inflammatory and erosive joint disease with a number of features in common with RA (28). In this report, we present data on the role of P-selectin in both the initiation and progression of CIA using mutant mice lacking the P-selectin protein. Interestingly, we found that the onset of arthritis was significantly accelerated, and the severity increased in P-selectin mutant mice, compared with wild-type. These studies show that P-selectin-mediated leukocyte adhesion is not required for the development of CIA, but P-selectin may play a regulatory role in controlling early leukocyte-dependent processes in the initiation and maintenance of immunologically driven joint inflammation.

Materials and Methods

Mice

Mice containing a null mutation for P-selectin were generated by gene targeting in 129/Sv embryonic stem cells, as previously described (5). The P-selectin mutation was then backcrossed onto the DBA/1 strain background for five generations. Control wild-type inbred DBA/1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice used were 12–16 wk of age, and routine serology tests were consistently negative for common viral pathogens.

Induction and assessment of arthritis

Arthritis was induced with chick type II collagen (Genzyme, Boston, MA), as previously described (29). Collagen was dissolved in 0.01 N acetic acid

5 Abbreviations used in this paper: RA, rheumatoid arthritis; CIA, collagen-induced arthritis.
DBA/1 mice were used as negative controls. Plates were read using a plate reader (Molecular Devices, Palo Alto, CA). Sera from nonimmunized mice were used in the assay. After washing, wells were incubated with goat anti-mouse IgG conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL) for 60 min at 37°C. This dilution was based on a titration of pooled serum from collagen-immunized DBA/1 mice, which gave submaximal color development in the assay. After washing, wells were incubated with goat anti-mouse IgG conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL) for 60 min at 37°C and developed with phosphatase substrate (p-nitrophenyl phosphate disodium; Sigma, St. Louis, MO) dissolved in diethanolamine buffer for 8 min. Color development was stopped with 3 M NaOH and absorbencies read at 405 nm using a microplate reader (Molecular Devices, Palo Alto, CA). Sera from nonimmunized DBA/1 mice were used as negative controls.

**Measurement of anti-type II collagen IgG**

Blood was obtained at various times by retro-orbital puncture under metofane anesthesia (Pitman-Moore, Mundelein, IL). Serum levels of anti-type II collagen IgG were measured by ELISA (29). Microtiter plates (96-well; Corning Costar, Cambridge, MA) were coated with 1 μg/well of chick type II collagen in bicarbonate buffer (pH 9.6) overnight at 4°C. After washing and blocking with 1% BSA, serum samples diluted 1/250 were added for 60 min at 37°C. This dilution was based on a titration of pooled serum from collagen-immunized DBA/1 mice, which gave submaximal color development in the assay. After washing, wells were incubated with goat anti-mouse IgG conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL) for 60 min at 37°C and developed with phosphatase substrate (p-nitrophenyl phosphate disodium; Sigma, St. Louis, MO) dissolved in diethanolamine buffer for 8 min. Color development was stopped with 3 M NaOH and absorbencies read at 405 nm using a microplate reader (Molecular Devices, Palo Alto, CA). Sera from nonimmunized DBA/1 mice were used as negative controls.

**Isolation and culture of splenocytes for cytokine production**

Spleens were removed from nonimmunized wild-type and P-selectin mutant mice and from type II collagen-immunized mice 14 days after immunization. Single cell suspensions were made and erythrocytes lysed with Tris-ammonium chloride. Cells were resuspended in RPMI 1640 containing 5% FBS, 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 1 mM sodium pyruvate (Life Technologies, Rockville, MD). Spleenocytes were cultured at 1.0 × 10^6/well in 200-μl volumes for 72 h at 37°C in quadruplicate wells and were either unstimulated or stimulated with plastic-adherent anti-mouse CD3 Ab (1 μg/well; 145-2C11; PharMingen, San Diego, CA). Cell culture supernatants were collected and IL-2, IFN-γ, IL-4, IL-5, and IL-10 measured by ELISA assays using anti-cytokine Abs and protocols supplied by PharMingen.

**Histological evaluation of joint tissue**

Joint tissue was collected from mice displaying the initial clinical signs of joint inflammation (0–5 days postonset, six mice each for wild-type and mutant groups) and from all mice at the end of each experiment, and fixed in 10% buffered formalin. Mouse paws were cut in half longitudinally, embedded in OCT (Miles, Kankakee, IL), and snap frozen in liquid nitrogen chilled iso-pentane. For histochemical staining, cryostat sections (10 μm) were air dried and run through a Diff-Quick staining procedure (Baxter, McGaw Park, IL). Briefly, slides were fixed with fixation solution (5 dips), washed in tap water to remove excess OCT, stained with solution 1 (eosin; 6 dips), tapped dry, stained with solution 2 (Thiazine Dye mixture, 6 dips), tapped dry, washed in tap water, air dried, and coverslipped with Permount (Fisher, Fair Lawn, NJ) for histopathological evaluation.

**Results**

**Incidence and onset of arthritis in P-selectin (-/-) mutant mice**

In three separate experiments, wild-type and P-selectin mutant DBA/1 mice were immunized with type II collagen and inspected daily for the development of arthritis. Surprisingly, the lack of expression of P-selectin did not suppress the development of arthritis, but actually led to an acceleration of arthritis. By day 28, a significant difference in the incidence of arthritis (as determined by χ^2 analysis; p < 0.001) was observed, as only 1/55 (2%) of wild-type mice showed clinical signs of arthritis, whereas 16/69 (23%) of P-selectin mutant mice had already developed arthritis (Fig. 1). On day 40, 12/55 (22%) of wild-type mice were arthritic, compared with 37/69 (54%) of P-selectin mutant mice (p < 0.001), and on day 70, the incidence was 28/55 (51%) and 51/69 (74%), respectively (p < 0.01). By the end of the studies, however, no significant differences were observed in the incidence of arthritis between the two groups (69% for wild-type, 77% for P-selectin mutants; p > 0.05). The day of onset in wild-type mice ranged from day 27 to day 91 with a median of 49 days postimmunization (mean = 52.9 ± 3.0 SEM). In contrast, the onset of arthritis in the P-selectin mutant mice was as early as day 14 (range 14–84), and the median onset, day 34, was 15 days earlier than the controls (mean = 37.9 ± 2.0). A cumulative number of arthritic mice in each group is given as a percentage.

**Number of involved paws and severity of arthritis**

In all experiments, arthritis P-selectin mutant mice had a consistently greater number of affected paws than the corresponding controls (data not shown), and the severity of arthritis was also increased in P-selectin mutant mice. As shown in Fig. 2, the severity scores in P-selectin mutant mice with arthritis were consistently higher than those in wild-type mice. The greatest differences were observed on days 50–91, where distortion and ankylosis progressed more rapidly in P-selectin mutant mice than in the wild-type controls.

**Anti-type II collagen Ab levels in wild-type and P-selectin mutant mice**

Serum IgG anti-type II collagen Ab levels were measured by ELISA at several time points postimmunization (Table I). No significant levels of Ab were detected on day 7 in either group. By day 14, however, Ab levels were readily detectable and were significantly higher in P-selectin mutant mice, compared with wild-type mice. This increase was not due to a difference in the relative incidence of arthritis, since none of the mice in these test groups had developed arthritis at the time of analysis. Significantly higher levels of Ab were also observed in nonarthritic P-selectin mutant mice on days 14 and 40, when compared with nonarthritic controls. A similar increase was observed when the Ab levels in arthritic P-selectin mutant mice were compared with arthritic...
controls on days 40 and 91. Ab levels on day 28 in arthritic P-selectin mutant and wild-type mice could not be statistically compared, since only one wild-type mouse was arthritic.

Cytokine profiles from splenocytes derived from wild-type and P-selectin mutant mice

Since Ig synthesis is driven by Th2-dependent cytokines, we determined whether the increased levels of anti-collagen IgG observed in both nonarthritic and arthritic P-selectin mutant mice were due to a shift in the relative balance of Th1 and Th2 cytokines. Spleen cells from both normal and type II collagen-immunized mice were cultured with or without anti-mouse CD3 Ab. The levels of IL-2, IFN-γ, IL-4, IL-5, and IL-10 were measured in the culture supernatants (Fig. 3). In contrast to wild-type mice, splenocytes from P-selectin mutant mice produced significantly less IL-2 (Fig. 3A). This difference was most pronounced in splenocytes from P-selectin mutant mice that had been immunized with type II collagen (a decrease of 84.8% of anti-CD3-stimulated IL-2 production, compared with wild-type mice). Production of IL-10 and IL-5, however, was comparable between the two groups of mice (data not shown). Spontaneous levels of IFN-γ were ~2.1–1.2 ng/ml, and anti-CD3-stimulated production ranged from 3.9 to 3.5 ng/ml.

Interestingly, both IL-10 and IL-5 synthesis induced by anti-CD3 were significantly increased in P-selectin mutant mice (Fig. 3, B and C). Augmentation of IL-10 production in both nonimmunized and immunized P-selectin mutant mice was increased 1.8- to 2.2-fold, and anti-CD3-induced IL-5 synthesis was elevated 1.9- to 2.8-fold, when compared with corresponding wild-type mice. No significant levels of spontaneous IL-10 or IL-5 synthesis were observed in any group. In contrast to the IL-10 and IL-5 data, IL-4 production in P-selectin mutant mice was decreased by 65% (non-immunized) and 74% (type II collagen-immunized), compared with wild-type mice (Fig. 3D).

Histology of arthritic joints from wild-type and P-selectin mutant mice

Joint tissue from arthritic wild-type and P-selectin mutant mice was assessed for the degree of leukocyte infiltration in forepaws and hindpaws. In mice displaying initial clinical signs of joint inflammation (0–5 days postonset), an intense inflammatory response was observed in periarticular soft tissues, but integrity of the joints remained intact, regardless of the severity of the inflammatory reaction. In both groups of mice, no obvious differences in the overall intensity of leukocytic infiltration between P-selectin mutant (Fig. 4A) and wild-type mice (data not shown) were observed. Gross inflammation of the fore and hindpaws (as assessed by clinical scoring) correlated well with marked infiltration of granulocytes (Gr-1+), the major cell type present, and macrophages (F4/80+) as defined by immunocytochemistry (data not shown). T lymphocytes (CD4+, CD8+ cells) were rarely seen in the lesions of either group (data not shown). Histologic examination of joint tissue from arthritic mice sacrificed at the end of each experiment revealed significant leukocytic infiltration, joint capsule thickening, bone destruction and/or bone remodeling, and ankylosis (Fig. 4B). No obvious differences in these parameters were observed between groups. We concluded that the P-selectin mutant mice exhibit the pathology expected in early and late CIA.

Discussion

Leukocyte/endothelial cell adhesion molecules are thought to play critical roles in the pathogenesis of several different inflammatory diseases, including RA (30, 31). Many different adhesion molecules, such as the selectins, have been shown to be expressed in chronically inflamed joints, suggesting that they mediate the cellular interactions in synovial tissue that lead to joint inflammation and destruction (31). To define more specifically the function of P-selectin in the development of chronic joint inflammation, we

Table I. Anti-type II collagen Ab levels in nonarthritic and arthritic mice

<table>
<thead>
<tr>
<th>Days After Immunization</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 40</th>
<th>Day 91</th>
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<table>
<thead>
<tr>
<th>Non-Arthritic</th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>P-selectin (+/+)</td>
<td>0.003 ± 0.002</td>
<td>1.655 ± 0.192</td>
<td>2.317 ± 0.126</td>
<td>1.765 ± 0.126</td>
<td>1.390 ± 0.134</td>
</tr>
<tr>
<td>n = 12</td>
<td>n = 11</td>
<td>n = 9</td>
<td>n = 7</td>
<td>n = 17</td>
<td></td>
</tr>
<tr>
<td>P-selectin (−/-)</td>
<td>0.004 ± 0.004</td>
<td>2.401 ± 0.207*</td>
<td>2.670 ± 0.132</td>
<td>2.400 ± 0.177*</td>
<td>1.665 ± 0.183</td>
</tr>
<tr>
<td>n = 9</td>
<td>n = 9</td>
<td>n = 6</td>
<td>n = 17</td>
<td>n = 16</td>
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<table>
<thead>
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<th>Arthritic</th>
<th></th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>P-selectin (+/+)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>2.314 ± 0.157</td>
</tr>
<tr>
<td>n = 6</td>
<td>n = 6</td>
<td>n = 6</td>
<td>n = 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-selectin (−/-)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>2.770 ± 0.074*</td>
<td>2.129 ± 0.084*</td>
</tr>
<tr>
<td>n = 20</td>
<td>n = 20</td>
<td>n = 20</td>
<td>n = 52</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean OD 405 nm ± SEM. No significant differences were observed between nonarthritic and arthritic mice for any time point. Significant differences between wild-type and mutant mice by Student’s t test are indicated: *, p < 0.05; #, p < 0.001; and #, p < 0.005.

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analyzed P-selectin mutant (−/−) mice in the CIA model. CIA is an accepted model of inflammatory joint disease with relevance to RA and has been used to address the role of various inflammatory mediators in arthritis (28, 32).

We found that the development of CIA was not inhibited in P-selectin mutant mice and that loss of P-selectin expression actually resulted in an acceleration and increased severity of the disease. These observations contrast to those reported in two different rat arthritis models. P-selectin Ab treatment of rats immunized with streptococcal cell wall proteins was shown to reduce both ankle edema and the number of infiltrating neutrophils into joints (33). In addition, anti-P-selectin Abs were found to inhibit both early (2 h) neutrophil and monocyte emigration into joint tissue, following induction of rat adjuvant arthritis (34). Our findings in the mouse CIA model suggest that either P-selectin expression on the synovial vasculature is not necessary for rolling and subsequent emigration of proinflammatory leukocytes into the joint in this model, or that other adhesion molecules can compensate for the loss of P-selectin expression and mediate leukocyte rolling. The absence of methods to directly quantify leukocyte rolling in synovial vessels does not allow discrimination between these two possibilities.

E- and L-selectin-mediated leukocyte rolling has been documented in cremaster muscle venules in P-selectin mutant mice following TNF-α treatment, and these adhesion molecules may be important for mediating proinflammatory leukocyte emigration into the synovium in this model (7, 35). Other adhesion molecules including VLA-4, VCAM-1, or CD44 may also mediate leukocyte rolling in synovial vessels. Treatment of mice with inhibitory Abs against CD44 has been shown to reduce joint swelling and synovial leukocyte emigration in established CIA and in proteoglycan-induced arthritis (36). VLA-4 Ab treatment of rats with adjuvant-induced arthritis also resulted in a reduction of leukocyte recruitment into joints (37–40). However, in both of these studies, it was not determined whether Ab treatment inhibited leukocyte rolling, firm

FIGURE 3. Analysis of in vitro cytokine production by splenocytes from P-selectin mutant and wild-type mice. Spleen cells from both nonimmunized P-selectin mutant and wild-type mice and from mice immunized with type II collagen for 14 days were cultured in vitro. Cells were left unstimulated (open bars) or were stimulated with anti-CD3 Ab bound to plastic for 72 h (shaded bars), and supernatant concentrations of IL-2 (A), IL-10 (B), IL-5 (C), IL-4 (D), and IFN-γ (data not shown) were determined by ELISA assay. Data shown are the mean ± SEM of quadruplicate assays on three to five mice per group. Significance by two-tailed Welch’s t test is indicated: +, p < 0.05; *, p < 0.005; **, p < 0.001.
adhesion, and/or transendothelial migration. Further studies of all adhesion molecules in both the CIA and other inflammatory models will be necessary to determine their role in the initial leukocyte/endothelial interactions within the synovial vasculature.

The accelerated arthritis and increased severity observed in P-selectin mutant mice was unexpected, and the mechanism(s) accounting for this observation is not clear. It is also not immediately obvious why P-selectin deficiency in this model results in increased production of anti-type II collagen Ab. Accelerated development of CIA has been reported in mice treated with anti-IL-10 Abs, IL-12, and in mice containing a null mutation in the IFN-γ receptor gene, suggesting that these cytokines play key roles in the initiation of joint inflammation in the CIA model (41–45). However, these studies did not determine the mechanisms by which loss or increased production of these cytokines led to the acceleration of arthritis.

Loss of P-selectin expression may selectively alter the trafficking patterns of specific lymphocyte subpopulations to peripheral lymphoid organs during the development of the immune response against type II collagen. This may result in differential production of cytokines, which may then directly or indirectly influence the inflammatory response in the joint. In our studies, we observed increased circulating titers of anti-type II collagen Abs in both arthritic and nonarthritic P-selectin mutant mice at several time points throughout these experiments (Table I). This correlated with significant increases in the production of the Th2 cytokines IL-10 and IL-5 from splenic lymphocytes in P-selectin mutant mice (Fig. 3). It is possible that the higher titers of circulating anti-type II collagen Ab in P-selectin mutant mice may contribute to the acceleration and significant increase in arthritis severity by augmenting immune complex deposition in the joint, thus leading to severe inflammation and joint destruction.

Another contributory factor to the accelerated onset and/or increased severity may be altered trafficking of a regulatory T cell subset into the joint during the initiation and/or progression of inflammation in P-selectin mutant mice. T lymphocytes play critical roles in the pathogenesis of both RA and CIA, and are thought to promote and inhibit the inflammatory response in the joint capsule through selective cytokine production (46–48). P-selectin has been shown to mediate rolling and emigration of specific T cell subtypes into tissue under various stimulation conditions. For example, P-selectin preferentially mediates the emigration of Th1 lymphocytes over Th2 lymphocytes into the skin during 2,4-dinitro-1-fluorobenzene (DNFB)-induced delayed-type hypersensitivity (49, 50). In addition, studies using in vitro flow chambers have shown that CD45RA+RO− (memory) T cells roll and bind more efficiently to Chinese hamster ovary cells expressing P-selectin than do CD45RA−RO+ (naïve) T cells, suggesting that P-selectin may influence their in vivo trafficking patterns (51, 52). Our data suggest that the role of P-selectin in T cell trafficking may be complex, since the acceleration of CIA cannot be easily explained by the inhibition of memory or Th1 regulatory cells into the joint.

It is also possible that the absence of P-selectin expression on synovial endothelium inhibits the recruitment of a suppressive monocyte population into the joint (53, 54). This could result in reduced levels of antiinflammatory mediators, such as TGF-β and IL-1ra, leading to accelerated inflammation (48, 55, 56). Inhibition or loss of P-selectin expression has been previously shown to reduce monocyte adhesion and emigration both in vivo and in vitro models of arthritis, as well as other inflammatory models (8, 14, 15, 27, 34). Although no obvious differences in the inflammatory infiltrates between arthritic P-selectin mutant and nonmutant mice were observed in swollen joints during the early phases of arthritis, alterations in the infiltrating leukocyte populations and/or cytokine production during the initiation of joint inflammation (before the onset of obvious joint swelling) may occur.

Our observations that P-selectin expression plays a regulatory role in the development of arthritis suggest that pharmacologic approaches designed to inhibit P-selectin might be detrimental for the treatment of inflammatory diseases, such as RA. These findings also suggest that variation in P-selectin expression among individuals could be a predisposing factor for the development of severe forms of inflammatory joint disease. Further studies of P-selectin in both RA and animal models of arthritis will be necessary to more specifically define its role in the initiation and progression of joint inflammation.

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References


