Synovial expression of osteopontin correlates with angiogenesis in juvenile idiopathic arthritis

M. Gattorno, A. Gregorio, F. Ferlito, V. Gerloni, A. Parafioriti, E. Felici, E. Sala, C. Gambini, P. Picco and A. Martini

Objective. To evaluate the synovial expression of osteopontin (OPN) and its possible correlation with the degree of synovial angiogenesis in human chronic idiopathic arthritis.

Methods. Forty-five patients with active juvenile idiopathic arthritis (JIA) were studied. All patients underwent SF aspiration before steroid injection. A paired plasma sample was collected from 22 JIA patients. Plasma from 15 age-matched healthy subjects was used as control. Plasma and SF were tested by ELISA for OPN and vascular endothelial growth factor (VEGF).

Synovial tissue was obtained at synovectomy from 10 JIA patients. Immunohistochemistry was performed according to a standard technique with anti-OPN, anti-CD68, anti-CD31 anti-VEGF and anti-\(\alpha_v\beta_3\) antibodies.

Results. OPN levels were significantly higher in SF than in paired plasma samples \((P < 0.001)\). The same pattern was observed for VEGF \((P < 0.001)\). A positive correlation between OPN and VEGF concentrations was found in SF \((r = 0.6, P = 0.001)\). In synovial tissue, OPN was expressed at the level of the lining and sublining layers with a distribution similar to that observed for VEGF. OPN expression in the lining layer correlated with the number of vessels present in the areas underlying the sublining layer.

Conclusions. Synovial expression of OPN correlates with parameters of angiogenesis in JIA. These data support, in human disease, the possible role of OPN in the vascularization of inflamed synovial tissue, as previously shown in OPN-deficient animal models of arthritis.

KEY WORDS: Osteopontin, Angiogenesis, Integrins, Juvenile idiopathic arthritis.
TABLE 1. Clinical and laboratory features of JIA patients at the moment of the serological study

<table>
<thead>
<tr>
<th>Polyarticular course*</th>
<th>Oligoarticular course</th>
</tr>
</thead>
<tbody>
<tr>
<td>(22 patients)</td>
<td>(23 patients)</td>
</tr>
<tr>
<td>Age (yr): mean (range)</td>
<td>8.2 (2.3–19.3)</td>
</tr>
<tr>
<td>Disease duration (yr)</td>
<td>3.1 (0.1–12.2)</td>
</tr>
<tr>
<td>No. of joints active/limited range of motion</td>
<td>5.1/5.0 (1–15)/0–16</td>
</tr>
<tr>
<td>PGI</td>
<td>5.9 (1–10)</td>
</tr>
<tr>
<td>ESR</td>
<td>48.2 (7–96)</td>
</tr>
<tr>
<td>Treatment</td>
<td>MTX (1 patient)</td>
</tr>
<tr>
<td></td>
<td>Enbrel, CS, MTX (2 patients)</td>
</tr>
<tr>
<td></td>
<td>NSAID, TX (5 patients)</td>
</tr>
<tr>
<td></td>
<td>NSAID alone (12 patients)</td>
</tr>
<tr>
<td></td>
<td>Cya, Ssz (1 patient)</td>
</tr>
</tbody>
</table>

PGI, physician global index; MTX, methotrexate; CS, corticosteroids; Cya, cyclosporin; Ssz, salazopyrin. *See patients and methods for disease onset.

Duran criteria [26] were studied. Twenty-two patients had a polyarticular course (four with systemic onset with a polyarticular course, five with RF-negative polyarticular onset, 13 with oligoarticular onset and extension to a polyarticular course) and 23 patients had a persistent oligoarticular course (Table 1). All patients had joint swelling (mostly of the knee) and underwent SF aspiration for steroid injection. A previous steroid injection in the same joint in the last 6 months was considered as an exclusion criterion.

A paired blood sample was collected with permission in 22 JIA patients (11 with polyarticular and 11 with oligoarticular course) and plasma was stored at −80°C immediately after centrifugation.

Clinical parameters (number of active joints, number of joints with limited range of motion and physician’s global assessment of overall disease activity) and laboratory parameters [ESR, CRP, white blood cell (WBC) and platelet (PLT) counts, serum concentration of haemoglobin (Hb)] of disease activity were recorded concomitantly.

Fifteen age-matched healthy subjects attending our clinic for preoperative examination for minor surgery (e.g. orchiopepy, phimosis correction) were used as controls after informed consent of the parents. A history of inflammatory or infectious disorders in the 4 weeks before the examination, together with clinical or laboratory (i.e. elevated ESR or CRP) signs of inflammation at the moment of the blood sampling, was considered an exclusion criterion.

Plasma and SF were tested with commercial ELISAs for OPN (Calbiochem, San Diego, CA, USA) and vascular endothelial growth factor (VEGF) (Amersham, UK) according to the manufacturers’ instructions.

Plasma levels of OPN and VEGF were compared between JIA patients and healthy controls using the non-parametrical Mann–Whitney U test. Correlations among all the variables considered were evaluated using the non-parametric Spearman rank test. Concomitant serum and SF determinations were evaluated with the Wilcoxon rank test.

**Immunohistochemical study**

Synovial tissues obtained at knee synovectomy from 10 JIA patients with an oligoarticular onset were studied (Table 2).

Eight specimens were fixed in 10% formalin for no longer than 24 h and subsequently embedded in paraffin. For two patients, frozen tissue was analysed for concomitant α,β1 and OPN determination. Immunohistochemical labelling was performed with a three-step immunoperoxidase technique using 3,3'-diaminobenzidine as the colour substrate, as described previously [27].

Primary monoclonal or polyclonal antibodies included anti-CD3 (Dako, Glostrup, Denmark), anti-OPN (Abcam, Cambridge, UK), anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-α,β1 (clone LM609; Chemicon, Temecula, CA, USA), anti-CD68 (clone KP1; Dako) and anti-CD31 (clone JC70A; Dako). Reactions in the absence of primary antibody and with irrelevant antibodies of the same isotypes (anti-cytomegalovirus, clones DDG9 and CCH2; Dako) were carried out as negative controls.

Quantitative evaluation of the slides was performed by two independent authors (MG and AG). The measurements for each antibody were performed on the same day. Ten high-power fields (hpf, 40×) with clear cellularity at the level of the lining and underlying sublining layers were evaluated for each patient. The number of positive cells and the number of vessels with an evident lumen were counted for each hpf. The means of the two independent evaluations were calculated and the final result was expressed as the number of positive cells or vessels/10 hpf.

**Table 2. Clinical characterization of JIA patients control at the moment of the immunohistochemistry study**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>JIA subgroup</th>
<th>Ongoing treatment</th>
<th>Source</th>
<th>No. of active joints</th>
<th>ESR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>6</td>
<td>Extended oligo</td>
<td>NSAID, MTX</td>
<td>Ankle</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>11</td>
<td>Persistent oligo</td>
<td>NSAID</td>
<td>Knee</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>11</td>
<td>Persistent oligo</td>
<td>MTX</td>
<td>Knee</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>8</td>
<td>Persistent oligo</td>
<td>MTX</td>
<td>Wrist</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>16</td>
<td>Persistent oligo</td>
<td>NSAID</td>
<td>Knee</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>12</td>
<td>Extended oligo</td>
<td>MTX</td>
<td>Knee</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>17</td>
<td>Extended oligo</td>
<td>MTX</td>
<td>Wrist</td>
<td>1</td>
<td>34</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>22</td>
<td>Persistent oligo</td>
<td>NSAID</td>
<td>Knee</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>12</td>
<td>Persistent oligo</td>
<td>NSAID</td>
<td>Knee</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>11</td>
<td>Persistent oligo</td>
<td>NSAID</td>
<td>Knee</td>
<td>1</td>
<td>13</td>
</tr>
</tbody>
</table>

MTX, methotrexate.
Correlation between OPN expression and vascularization in synovial tissue

OPN was clearly expressed at the level of the lining layer and, with a slightly lower intensity, in the sublining layer (Fig. 3). Serial sections stained with anti-CD68 monoclonal antibodies showed that OPN was expressed mainly by CD68-negative (fibroblast-like) cells, but also by CD68-positive cells (Fig. 3E and H). No expression was found in the lymphocytic aggregates (not shown). Notably, clear positivity was also found at the level of extracellular matrix surrounding areas of intense vascularization (Fig. 3D).

All synovial specimens showed the presence of intense vascularization, mainly at the level of sublining layers (Fig. 3C and F). Diffuse lymphocytic infiltrates were present in all patients, whereas follicular-like structures with small vessels in them were found in three specimens only.

VEGF was abundantly expressed at the level of both the lining and the sublining layer (Fig. 3B and I). Clear positivity was found mainly on CD68-positive cells, but also on fibroblast-like (CD68-negative) cells and on CD31-positive endothelial cells (Fig. 3E and I). Some VEGF-positive cells were also found at the level of lymphocytic aggregates (not shown).

The total numbers of vessels/positive cells per 10 hpf in the lining and sublining layers for the main studied markers for each patient are given in Table 3.

The expression of OPN at the level of the lining layer showed a marked positive correlation with the expression of OPN ($r = 0.74$, $P = 0.03$) and the number of vessels in the corresponding sublining areas ($r = 0.71$, $P = 0.04$). On the contrary, the number of OPN-positive cells did not correlate with the numbers of CD68- and CD3-positive cells in either the lining layer or the sublining layer. The number of cells positive for VEGF at the level of lining layer showed a positive correlation with the number of VEGF-positive cells in the corresponding sublining layer ($r = 0.78$, $P = 0.02$). Moreover, the expression of VEGF at the level of the sublining layer correlated positively with the number of CD68-positive cells ($r = 0.76$, $P = 0.02$) and with the number of vessels ($r = 0.76$, $P = 0.02$) in the same areas.

No significant correlation was found between OPN and VEGF at the level of either the lining or the sublining layer. Serial frozen sections from two additional patients were stained for OPN and one of its ligands, $\alpha_v\beta_3$ integrin. In some areas, close anatomical proximity was found between OPN expressed in the lining layer and $\alpha_v\beta_3$ integrin-positive endothelial cells of the underlying areas (Fig. 3L–O).

Discussion

The aim of the present study was to evaluate, in the setting of human chronic inflammatory arthritis, the possible correlation between the synovial expression of OPN and angiogenesis.
Fig. 3. (A–D) Expression of (A) CD68, (B) VEGF, (C) CD31 and (D) OPN in patient 2 (Table 2) (4×). Lining (LL) and sublining (SLL) layers are shown in A and C. Expression of OPN at the level of lining layer (black arrow) and in the perivascular extracellular matrix (white arrow) is shown in D. (E–I) Consecutive slides showing the expression of (E) CD68, (F) CD31, (G) CD3, (I) VEGF and (H) OPN in patient 6 (Table 2) at high magnification (20×). (L–O) Expression of (L) OPN and (M) integrin αvβ3 in frozen synovial tissue from patient 9 (Table 2) (10×). Panel N shows the area of the box in L at higher magnification (40×), and demonstrates OPN expression at the level of the lining layer. Similarly, in O the expression of αvβ3 on endothelial cells of the same area is shown at higher magnification (40×).
Osteopontin and angiogenesis in JIA

Angiogenesis is a physiological process consisting in the formation of new blood vessels from the pre-existing microvascular bed. The regulation of angiogenesis is strictly modulated by a balance between angiogenic (angiogenin, cytokines, growth factors) and angiostatic (angiostatin, endostatin, thrombospondin, placental ribonuclease inhibitor) factors [28].

Pro-angiogenic mediators induce the production by endothelial cells of proteolytic enzymes (metallloproteinases, plasminogen activator, proteases) that stimulate the degradation of the venule basement membrane [29]. This process is followed by the migration and proliferation of stimulated endothelial cells and the subsequent formation of new vessels [30].

In chronic synovitis, new vessel growth provides the oxygen and nutrients necessary for the high metabolic requirement of synovocytes and allows the migration and progressive infiltration of other inflammatory cells into the joint [31]. VEGF has been shown to be one of the most relevant pro-angiogenic factors in the synovial environment, both in adult RA [32, 33] and in JIA [34, 35].

The role of OPN in angiogenesis has been stressed by many in vitro and in vivo studies. The engagement of integrins (mainly αβ3 and αβ5) expressed on endothelial cells with their ligands (including OPN) promotes the proliferation and migration of the latter cells [14, 24, 25, 36]. Moreover, the integrin-OPN interaction prevents endothelial cells from undergoing apoptosis through a nuclear factor κB (NF-κB)-mediated mechanism [37]. In a recent in vitro study, a possible integrin-independent mechanism of OPN-driven neovascularization was also suggested [38].

In the present study we analysed the expression of OPN in synovial fluids and synovial membranes from JIA patients in order to detect possible correlations between the expression of OPN and some parameters of angiogenesis, such as the concentration of VEGF and the number of vessels in synovial tissue.

As expected, synovial concentrations of OPN and VEGF were elevated and significantly higher than in paired plasma. Interestingly, synovial fluid OPN concentrations showed a clear positive correlation with VEGF, and these two molecules showed a similar tissue distribution. These data are in line with the close functional cooperation that has been described between VEGF and OPN in the control and stimulation of angiogenesis [14, 24].

Immunohistochemistry showed a clear positive correlation between the expression of OPN and the degree of vascularization in the corresponding areas of the sublining layer. Conversely, OPN did not show any significant correlation with macrophages and lymphocytes, for which OPN has been described to exert chemotactic activity [8–10].

According to many authors, αβ3 is a reliable marker for endothelial cells undergoing a process of neoangiogenesis [36, 39]. Interestingly, OPN-positive cells of the lining layer showed close anatomical proximity with αβ3 expressed on the endothelial cells. This suggests that, at least in the context of JIA synovium, the OPN-induced stimulation of angiogenesis could be mediated mainly by interaction with the integrins expressed on endothelial cells [36–38].

Altogether, these data support the hypothesis that stimulation of angiogenesis via integrin engagement may be one of the most relevant regulating properties of OPN in the context of inflammation. This agrees with the findings of Yumoto et al. [23] that OPN deficiency protects joints against destruction in anti-type II collagen antibody-induced arthritis in mice. Indeed, in their model the protective role of OPN deficiency against cartilage destruction was mainly related to decreased synovial angiogenesis and reduction of chondrocyte apoptosis.

Interestingly, an in vivo study has shown that even the role of OPN in bone resorption is mainly related to the regulation of vascularization of the bone by haemangiogenic endothelial cells, leading to subsequent osteoclast accumulation [40].

In conclusion, our findings support the hypothesis of a close functional relationship between synovial expression of OPN and the degree of angiogenesis in human chronic synovitis, and further suggest that an anti-angiogenic treatment based on blockade of interactions between integrins expressed on endothelial cells and their ligands may be an option in the treatment of human arthritis [41–43].

Table 3. Expression of OPN and VEGF in synovial tissue and correlation with macrophage (CD68) and lymphocyte (CD3) infiltration and with the number of vessels in the corresponding areas

<table>
<thead>
<tr>
<th>Patient</th>
<th>OPN*</th>
<th>VEGF*</th>
<th>CD68*</th>
<th>CD3*</th>
<th>Vessels†</th>
<th>OPN*</th>
<th>VEGF*</th>
<th>CD68*</th>
<th>CD3*</th>
<th>Vessels†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>434</td>
<td>915</td>
<td>500</td>
<td>140</td>
<td>0</td>
<td>608</td>
<td>1406</td>
<td>405</td>
<td>802</td>
<td>169</td>
</tr>
<tr>
<td>2</td>
<td>251</td>
<td>943</td>
<td>980</td>
<td>230</td>
<td>0</td>
<td>2023</td>
<td>1728</td>
<td>525</td>
<td>1166</td>
<td>230</td>
</tr>
<tr>
<td>3</td>
<td>159</td>
<td>1094</td>
<td>977</td>
<td>182</td>
<td>0</td>
<td>78</td>
<td>1639</td>
<td>959</td>
<td>874</td>
<td>134</td>
</tr>
<tr>
<td>4</td>
<td>283</td>
<td>833</td>
<td>915</td>
<td>270</td>
<td>0</td>
<td>198</td>
<td>1506</td>
<td>588</td>
<td>1128</td>
<td>215</td>
</tr>
<tr>
<td>5</td>
<td>140</td>
<td>1092</td>
<td>752</td>
<td>6</td>
<td>0</td>
<td>148</td>
<td>1404</td>
<td>484</td>
<td>1144</td>
<td>130</td>
</tr>
<tr>
<td>6</td>
<td>276</td>
<td>812</td>
<td>751</td>
<td>25</td>
<td>0</td>
<td>134</td>
<td>945</td>
<td>420</td>
<td>488</td>
<td>178</td>
</tr>
<tr>
<td>7</td>
<td>106</td>
<td>400</td>
<td>844</td>
<td>84</td>
<td>0</td>
<td>24</td>
<td>568</td>
<td>356</td>
<td>990</td>
<td>74</td>
</tr>
<tr>
<td>8</td>
<td>185</td>
<td>718</td>
<td>770</td>
<td>70</td>
<td>0</td>
<td>148</td>
<td>923</td>
<td>470</td>
<td>1353</td>
<td>123</td>
</tr>
</tbody>
</table>

*Number of positive cells/10 high-power fields; † number of vessels/10 high-power fields.

See text for statistical correlations.

**Key message**

- The expression of osteopontin in synovial environment is closely related to the degree of angiogenesis.

Acknowledgement

We would like to thank Professor R. Ravazzolo for stimulating discussions on the role of OPN in human diseases.

The authors have declared no conflicts of interest.
References


