EXTENDED REPORTS

Increased cellularity and expression of adhesion molecules in muscle biopsy specimens from patients with rheumatoid arthritis with clinical suspicion of vasculitis, but negative routine histology

Patrick C Verschueren, Alexandre E Voskuyl, T J Smeets, Koos H Zwinderman, Ferdinand C Breedveld, Paul P Tak

Abstract

Objective—Histological analysis of random quadriceps muscle biopsy specimens can be used to detect vasculitis in patients with rheumatoid arthritis (RA). This study aimed at determining the immunohistological features in patients with clinical suspicion of rheumatoid vasculitis, but without a transmural infiltrate or fibrinoid necrosis of the vessel wall on routine histology.

Methods—Three groups of patients with RA were studied: (a) without clinical signs of vasculitis (n=6); (b) with recent onset of extra-articular features and a clinical suspicion of vasculitis but normal routine histology (n=11); and (c) with recent onset of extra-articular features and vasculitis, histologically proved either in muscle or other biopsy specimens (n=14). A control group of patients with osteoarthritis was also included (n=5). Frozen sections from quadriceps muscle biopsy specimens were analysed with monoclonal antibodies to detect CD3, CD4, CD8, CD68, ICAM-1, VCAM-1, and HLA-DR. The slides were evaluated using a semiquantitative scoring system (0–4).

Results—The mean scores gradually increased from group 1 to 3, leading to significant differences between groups 1 and 2, but not between groups 2 and 3 for most markers (p< 0.05). Thus the pathological changes were similar for the two groups with clinical signs of vasculitis, even when the conventional histological evaluation was negative. Higher immunohistological scores were associated with perivascular infiltrates on routine histology.

Conclusion—The pathophysiological events leading to vasculitis are reflected by the changes in the quadriceps muscle biopsy specimens. The data indicate that the sensitivity of examination of muscle biopsy specimens for the diagnosis of rheumatoid vasculitis can be increased by the use of new criteria.

In patients with rheumatoid arthritis (RA), vasculitis is a complication with several clinical presentations, which can be sometimes difficult to diagnose. Inflammation of small and medium sized vessels can cause skin disorders such as rash, cutaneous ulcerations and gangrene, neuropathy, eye symptoms, and may also affect visceral organs.

The clinical picture is of help in the diagnostic process, but most clinicians rely on histology for the diagnosis. Biopsy specimens can be obtained from clinically affected organs whenever these are accessible—for instance, in cases of peripheral neuropathy, but often randomly obtained biopsy specimens of skeletal muscle are used as an alternative. Other possibilities include rectum or labial salivary gland biopsy specimens. The histological gold standard for the diagnosis of vasculitis is the presence of a transmural infiltrate or fibrinoid necrosis of the vessel wall. In skin biopsy specimens leucocytoclasia is used as a diagnostic criterion.

The sensitivity for the diagnosis vasculitis is limited (<50%) when randomly obtained muscle biopsy specimens are examined owing to the segmental distribution of the histological abnormalities and, usually, the restricted organ involvement. The diagnostic yield of muscle samples can be improved by examining multiple biopsy sections and by using new diagnostic criteria for vasculitis, such as the presence of perivascular infiltrates in more than three cell layers. This criterion is more specific and equal for the diagnosis of rheumatoid vasculitis, when compared with the criteria described above.

In addition, immunohistological techniques might offer diagnostic possibilities beyond conventional morphological examination by quantification of certain functional aspects of the structures involved.
This study aimed at examining the cell infiltrate and the expression of adhesion molecules in muscle biopsy specimens from patients with rheumatoid vasculitis in order to determine whether immunohistological analysis may have an additional diagnostic value compared with routine histological examination.

**Methods**

**PATIENTS**

Three groups of patients with RA were selected. Firstly, patients without any clinical sign of vasculitis were included. In this so-called “RA−” group the mean age of the six patients with RA was 72 years, with a mean disease duration of 12 years. Rheumatoid factors were detectable in five out of six, all had erosive disease, and none had subcutaneous nodules. Four of the patients in this group were treated with disease modifying antirheumatic drugs (DMARDs) (three sulfasalazine and one hydroxychloroquine) and one patient used prednisone (13 mg/d).

Secondly, patients with recent onset of extra-articular features (<6 months) but no histological proof of vasculitis were included. This “RA+” group comprised 11 patients with RA with a mean age of 65 years and a mean disease duration of 19 years. All were rheumatoid factor positive and had erosive disease. A majority had subcutaneous nodules (6/11). Of the patients in this group eight of 11 used DMARDs (three sulfasalazine, three intramuscular gold, one methotrexate, and one azathioprine). Two patients used prednisone (10 mg/d). The mean swollen joint count in this group was 10.6 (SD 4.0) and the mean tender joint count 13.3 (SD 9.3).

Thirdly, patients with recent onset of extra-articular features and histological proof of vasculitis were included. This so-called rheumatoid vasculitis (“RV”) group consisted of 14 patients with RA with a mean age of 71 years and a mean disease duration of 15 years.

All patients were rheumatoid factor positive, most of them had erosive disease (13/14), and a majority had subcutaneous nodules (9/14). In this group eight of 14 patients used DMARDs (six sulfasalazine and three hydroxychloroquine) and four of 14 used prednisone (three 10 mg/d, one 40 mg/d). In the subgroup of patients in which vasculitis was proved by quadriceps muscle biopsy (n=7), the mean swollen joint count was 7.4 (SD 4.4) and the tender joint count 8.7 (SD 6.7). In the subgroup of patients (n=7) with histological confirmation of vasculitis in other biopsy specimens, the mean swollen joint count was 10 (SD 2.5) and the mean tender joint count 9.3 (SD 3.0).

The extra-articular manifestations of RA were mainly nailfold lesions, purpura, cutaneous ulcers, and distal sensorimotor or sensory polyneuropathy. Weight loss was a common extra-articular feature, but it was almost always accompanied by other extra-articular manifestations of RA. Table 1 gives details of the extra-articular feature, but it was almost always accompanied by other extra-articular manifestations of RA. Table 1 gives details of the extra-articular feature, but it was almost always accompanied by other extra-articular manifestations of RA. Table 1 gives details of the extra-articular feature, but it was almost always accompanied by other extra-articular manifestations of RA. Table 1 gives details of the extra-articular feature, but it was almost always accompanied by other extra-articular manifestations of RA.
IMMUNOHISTOLOGICAL ANALYSIS

A part of each quadriceps muscle sample was snap frozen in Tissue-Tek OCT (Miles Inc, Elkhart, IN) by immersion in methylbutane (−70°C). Only quadriceps muscle sections were used for the immunohistological investigations, even when by routine histology vasculitis was shown only in tibial muscle, skin, or sural nerve biopsy samples.

Frozen blocks were stored at −70°C until sectioned for immunohistological staining. Serial sections of 7 μm were cut on a cryostat and placed on glass slides (Star Frost adhesive slides, Knittelgläser, Germany).

Slides were fixed in acetone at room temperature for 10 minutes. Sections were stained with the following monoclonal antibodies: anti-CD3 (Becton-Dickinson, San Jose, CA), anti-CD4 (Becton Dickinson), anti-CD8 (Dako, Glostrup, Denmark), anti-CD68 (Dako), anti-ICAM-1 (MEM-111, Sandbio), anti-VCAM-1 (1G11B1, Sandbio), and anti-HLA-DR (Dako).

After incubation with the primary antibody, affinity purified, horseradish peroxidase conjugated goat-antimouse (Dako) and swine-antigoat antibodies (Tago, Burlingame, CA) were used for detection of the cell markers and adhesion molecules according to the three step immunoperoxidase method, as described previously.15

In addition, a subdivision between patients with or without perivascular infiltrates of more than three cell layers on routine histology was made in the RA+ and the RV group. The mean scores for each marker were compared between the patient groups and statistical significance was evaluated with variance comparison.16 17 All biopsy specimens were blinded and scored by two independent observers according to this validated semi-quantitative scoring system.

Scores never differed by more than one point between observers. Whenever there was initial disagreement on a particular score a consensus score was determined.

STATISTICAL ANALYSIS

The mean scores for each marker were compared between the patient groups and statistical significance was evaluated with variance analysis and Student’s t tests. A separate comparison between the RA+ and the RV group was made to check whether immunohistology might be of any value for differential diagnosis.

In addition, a subdivision between patients with or without perivascular infiltrates of more than three cell layers on routine histology was made in the RA+ and the RV group. The mean immunohistological scores of these subgroups were compared separately.

Results

After the routine histological examination seven patients were assigned to the RV group on the basis of a positive quadriceps biopsy, two were assigned to this group on the basis of a positive sural nerve biopsy, and two were assigned to this group on the basis of clinical signs of vasculitis without proof on routine histology (RA+), patients with RA with clinical signs of vasculitis (RA−), patients with RA suspected of vasculitis (RA+), and patients with RA with histologically proved vasculitis (RV). Values are the mean semiquantitative scores (standard error)

<table>
<thead>
<tr>
<th>Marker</th>
<th>OA (n=5)</th>
<th>RA− (n=6)</th>
<th>RA+ (n=11)</th>
<th>RV (n=14)</th>
<th>RA+ (n=11)</th>
<th>RA− (n=6)</th>
<th>OA (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>0.6 (0.2)</td>
<td>0.5 (0.2)</td>
<td>1.7* (0.2)</td>
<td>2* (0.3)</td>
<td>0.6 (0.2)</td>
<td>0.5 (0.2)</td>
<td>CD3</td>
</tr>
<tr>
<td>CD4</td>
<td>0.6 (0.2)</td>
<td>0.7 (0.2)</td>
<td>1.5* (0.2)</td>
<td>2* (0.3)</td>
<td>0.6 (0.2)</td>
<td>0.7 (0.2)</td>
<td>CD4</td>
</tr>
<tr>
<td>CD8</td>
<td>0.4 (0.2)</td>
<td>0.2 (0.2)</td>
<td>1.3* (0.3)</td>
<td>2.6† (0.3)</td>
<td>0.4 (0.2)</td>
<td>0.2 (0.2)</td>
<td>CD8</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>1 (0)</td>
<td>1.2 (0.3)</td>
<td>2.3* (0.2)</td>
<td>2.7† (0.3)</td>
<td>1 (0)</td>
<td>1.2 (0.3)</td>
<td>ICAM-1</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>1 (0)</td>
<td>1.2 (0.2)</td>
<td>2* (0.3)</td>
<td>2.7† (0.3)</td>
<td>1 (0)</td>
<td>1.2 (0.2)</td>
<td>VCAM-1</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>1 (0)</td>
<td>0.2 (0.2)</td>
<td>1.2 (0.3)</td>
<td>1.9† (0.3)</td>
<td>1 (0)</td>
<td>0.2 (0.2)</td>
<td>HLA-DR</td>
</tr>
</tbody>
</table>

* p<0.05 (Student’s t test for differences between RA− and RA+).
† p<0.05 (Student’s t test for differences between RA− and RV).

The difference between RA+ and RV was not statistically significant.

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Figure 1A  CD3+ T cells.

Figure 1B  CD4+ cells.
Figure 1C  CD8+ cells.

Figure 1D  CD68+ macrophages.
Figure 1E  Adhesion molecule ICAM-1.

Figure 1F  Adhesion molecule VCAM-1.
a positive musculus tibialis biopsy, one on the basis of a positive nervus suralis biopsy, and four on the basis of a positive skin biopsy. Vasculitis was found concomitantly in a quadriceps muscle biopsy specimen as well as in a skin biopsy sample in four cases, and in a quadriceps muscle biopsy specimen as well as in a musculus tibialis biopsy sample in one case (table 1). Patients assigned to the RA+ group did not show any sign of fibrinoid necrosis on routine histology, either in the quadriceps muscle sample, which was taken in all of them, or in additional biopsy specimens. During the routine histological examination of the quadriceps muscle biopsy specimen perivascular infiltrates were documented in 11 of 14 patients in the RV group and in four of 11 patients in the RA+ group. No perivascular infiltrates were seen in the RA− and the OA group. A few immunohistological slides could not be evaluated owing to the poor quality of the tissue. These included one ICAM-1 slide in each patient group, two CD8 slides in the RA+ and one in the RV group, and one CD4 slide in the RA+ group. All other sections were of good quality. A gradual increase in the mean immunohistological scores was recorded from group 1 (RA−), to group 2 (RA+), to group 3 (RV) (table 2). The scores in the OA control group were similar to the RA− group. (Original magnification × 400.)

Generally, patients with perivascular infiltrates on routine histology had significantly higher mean immunohistological scores for all markers than those without such infiltrates (fig 2).
Muscle biopsy specimens from patients with RA

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*p<0.05 (Student’s t test comparing PVI− and PVI+).

Adhesion molecules are upregulated by cytokines21 and have an important role in the histology compared with the RA+ group. Several clinical18–20 and experimental studies20 suggest that rheumatoid vasculitis is caused by circulating immune complexes containing rheumatoid factor and autoantibodies such as anti-endothelial cell antibodies, forming deposits in vessel walls and triggering an inflammatory reaction, which may lead to endothelial cell injury. Apart from these humoral determinants, the morphological changes associated with the acute phase of vasculitis suggest a pathogenetic role for polymorphonuclear and mononuclear cells.20

Adhesion molecules are upregulated by cytokines21 and have an important role in transendothelial migration of leucocytes.

The levels of circulating ICAM-1 and ICAM-3 are raised in patients with rheumatoid vasculitis compared with patients with RA without vasculitis.22

The accumulation of activated cells and upregulated expression of adhesion molecules described here also reflect the pathophysiological events leading to vasculitis. It remains to be elucidated whether there is a difference, between symptomatic patients with histological proof according to the conventional vasculitis classification and those without. The higher degree of inflammation seen in the first group suggests that it might be only a matter of quantity. This could have therapeutic implications, as most clinicians rely on histology for the diagnosis of rheumatoid vasculitis and, consequently, their treatment strategies rely on histology as well.

Taken together, these results confirm and extend our previous study, suggesting changes on the vascular endothelium as well as perivascular cell infiltration in random quadriceps muscle biopsy specimens of patients with RA with clinical signs of vasculitis, regardless of histological proof according to the conventional vasculitis classification. The data indicate that the sensitivity of the examination of random muscle biopsy samples for the diagnosis of rheumatoid vasculitis can be increased by the use of new criteria. The ultimate value of these observations for differential diagnosis remains to be determined in larger clinical studies.


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