Increased endothelial expression of HLA-DQ and interleukin 1α in extra-articular rheumatoid arthritis. Results from immunohistochemical studies of skeletal muscle

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Abstract

Objective. To investigate markers of endothelial activation in muscle biopsies from rheumatoid arthritis (RA) patients with and without extra-articular manifestations (ExRA).

Patients and methods. Nine consecutive ExRA patients were compared with nine RA controls without ExRA, matched for age, sex and duration of RA. Muscle biopsies were obtained from the lateral vastus or anterior tibial muscle. Macrophage and lymphocyte CD markers, HLA molecules, cytokines and adhesion molecules were investigated using immunohistochemistry, and stainings were evaluated using computer image analysis and conventional microscopy. Serum concentrations of soluble adhesion molecules, tumour necrosis factor α (TNF-α) and rheumatoid factor (RF) were determined using immunoassays.

Results. The number of HLA-DQ-positive capillaries (P = 0.039) and the expression of interleukin 1α (IL-1α) in endothelial cells (mean pairwise difference 0.26%; 95% confidence interval 0–0.52) were increased in ExRA patients compared with non-ExRA controls. There were no signs of inflammatory cell infiltrates or fibre degeneration. Serum levels of TNF-α, the soluble form of intercellular adhesion molecule 1, the soluble form of vascular cell adhesion molecule 1 and IgM RF were increased in the ExRA group.

Conclusion. The increased expression of HLA-DQ and IL-1α may indicate systemic endothelial activation in extra-articular RA, which could be of importance for cardiovascular comorbidity and mortality in such patients.

KEY WORDS: Rheumatoid arthritis, Extra-articular manifestations, Endothelial activation, Interleukin-1α, HLA-DQ, Cardiovascular disease, Vasculitis.

Rheumatoid arthritis (RA) is a chronic disease characterized by inflammatory polyarthritis, which in some cases is complicated by extra-articular manifestations (ExRA). The latter range from mild signs of serositis, which may only be detectable at autopsy [1], to life-threatening necrotizing vasculitis involving internal organs [2]. In a well-defined RA population, the majority of clinically evident ExRA is made up of cases of pleuritis, pericarditis and cutaneous vasculitis [3]. The systemic nature of RA is further underlined by the presence of fever and weight loss in severe cases [4]. RA has consistently been found to be associated with an increased mortality rate compared with the general population [5–7], and most of the excess mortality is due to cardiovascular deaths [7, 8]. The mortality among patients with ExRA has been found to be even higher than among non-extra-articular RA patients [3, 9, 10]. A subset of ExRA patients with a poor prognosis has been defined [3, 11], and cardiovascular mortality appears to be particularly increased in this subgroup [3]. The mechanism behind the increased risk of cardiovascular death in RA and in ExRA in particular is not known, but chronic inflammation has been suggested as
a risk factor for cardiovascular death in RA and ExRA; this is probably related to atherosclerotic vascular disease [8]. Soluble forms of intercellular adhesion molecule 1 (ICAM-1) and other cellular adhesion molecules have been detected in serum in increased amounts in patients with RA [12] as well as in other chronic diseases with established blood vessel involvement, such as systemic vasculitis [13] and systemic sclerosis [14]. In a comparison of RA patients with and without vasculitis, circulating ICAM-1 concentrations were significantly higher in the vasculitis subgroup [15]. In addition, in longitudinal studies of apparently healthy men [16] and women [17], the soluble form of ICAM-1 (sICAM-1) was found to be an important predictor of cardiovascular disease. These findings are compatible with generalized blood vessel involvement in at least subgroups of RA patients, and could be important in the pathogenesis of cardiovascular disease.

Muscle tissue is well suited to the investigation of blood vessels, as it is easy to biopsy and the muscle tissue from the extremities usually contains both microvessels and small arteries [18]. Vascular involvement in skeletal muscle in ExRA has been reported, including necrotizing arteritis [19] in a small number of patients with muscular symptoms. Vasculitis has also been reported in striated muscle biopsies from RA patients who clinically presented signs of vasculitis in other organs (e.g. neuropathy and cutaneous lesions) [20, 21], and a pattern of increased expression of adhesion molecules and HLA-DR in muscle tissue has been reported from such patients without histopathological evidence of vasculitis [22].

The present study was undertaken in order to investigate whether there is more generalized endothelial activation in patients with extra-articular manifestations of RA than in non-extra-articular RA patients. We studied the expression of markers of endothelial activation and inflammation in skeletal muscle tissue and in serum from RA patients with and without extra-articular manifestations.

Materials and methods

Patients

Nine patients with previously diagnosed RA, all fulfilling the 1987 American College of Rheumatology classification criteria for RA [23], who developed extra-articular manifestations according to predefined criteria [3, 11, 37] were included in the study. The patients were recruited from rheumatology out-patient clinics in Malmö (n = 8) and Ängelholm (n = 1). Their ExRA consisted of pleuritis (n = 2), pericarditis (n = 2), Felty’s syndrome (n = 2) or cutaneous vasculitis (n = 3). Briefly, in the patients with pericarditis and pleuritis, clinically suspected exudation was verified by echocardiography and chest X-ray respectively. The patients with Felty’s syndrome had splenomegaly by ultrasound measurement, neutrophils < 1.8 × 10^9/L on at least two occasions, and no other probable explanation of the neutropenia. Cutaneous vasculitis was diagnosed by biopsy (n = 2) or clinical judgement by a dermatologist (n = 1). Arterial or venous insufficiency to the extent that could explain the lesions was not present. All cases were examined by the same physician (CT) before inclusion. RA controls without ExRA, matched for age, sex and duration of RA, were selected from a database (established in 1997) containing all known RA patients attending out-patient clinics run by rheumatologists in Malmö. Only controls fulfilling the 1987 American College of Rheumatology criteria for RA [23] were included. Previous and current absence of ExRA (according to the same criteria as the cases, in addition including the absence of rheumatoid nodules) in the controls was verified by evaluation of medical records, a detailed questionnaire and physical examination. Patients with previous or current rheumatoid nodules were excluded, as nodules have been shown to predict severe extra-articular disease [37] and inclusion of nodular patients in the non-ExRA group would put them at risk of developing ExRA. All cases and controls were examined by the same physician (CT), using a structured protocol. ExRA cases and non-ExRA controls were well matched for sex (three men and six women in each group), age [ExRA, median 61 yr; interquartile range (IQR) 58–69 yr; non-ExRA, 59 yr, 54–66 yr] and duration of RA (ExRA, median 15 yr, IQR 6–23; non-ExRA 17 yr, 7–20 yr). There was no major difference in the number of current smokers (ExRA 2, non-ExRA 1), previous smokers (ExRA 6, non-ExRA 5) or in systolic blood pressure (ExRA, median 130, IQR 120–140; non-ExRA 130, 120–145). There was no substantial difference in the number of individuals previously positive for RF (78% in both groups) or antinuclear antibodies (ExRA 38%, non-ExRA 25%). ExRA patients tended to be on glucocorticoid treatment (22 vs 56%) (P = 0.33) and treatment with disease-modifying anti-rheumatic drugs (DMARDs) (56 vs 100%) (P = 0.082) to a lesser extent than non-ExRA controls at the time when they developed the ExRA manifestation. The patients with ExRA tended to have a higher number of swollen joints [median 8, IQR 5–19 vs 2 (0–4)] (P = 0.09), higher scores for Ritchie’s index (median 16, IQR 8–19 vs 5, 2–8) (P = 0.42) and higher levels of C-reactive protein (CRP) (median 31 mg/l, IQR 13–64 vs 10, 0–21) (P = 0.12).

None of the RA patients had muscle pain, weakness or other clinical signs of muscle disease. All patients gave their informed consent to participation and the study was approved by the ethics committee of Lund University.

Tissue samples

Muscle biopsy was performed before any changes in pharmacological treatment were made due to the extra-articular manifestations. Biopsies from the vastus lateralis or the tibialis anterior muscle were obtained from each patient under local anaesthesia using a semi-open technique with a conchotome [24]. At least two biopsy samples were taken from each patient,
Quantitation of stained cells
The tissue sections were analysed with a Reichert–Jung Polyvar microscope. Quantitation was performed both by conventional microscopy and by computer-assisted imaging analysis. For the conventional reading, the numbers of positively stained mononuclear cells and endothelial cells of the whole tissue section were estimated. The origin of the muscle biopsies was concealed to the investigators during the quantitation. The sections were read on different occasions by two of the authors (CT and IEL) with concordant results. Mean values of these readings were divided by the total tissue area of the biopsy, measured by computer image analysis (see below).

The numbers of mononuclear cells per unit area of cryocut section stained positively for the markers CD3, CD4, CD8, CD68 and CD163 in combination, CD19 and CD20 in combination, LFA-1 and VLA-4 were determined in the same way. The number of cells and vessels stained positively for VCAM-1, ICAM-1 and HLA-DQ were scored separately; only the number of positive vessels is given in the results as no extra information was derived from this subdivision. For HLA-ABC and HLA-DR staining, a 5-point scale was used to score the percentage of positively stained muscle fibre area relative to the total section area: 0 = positive staining in endothelial cells, negative muscle fibres; 1 + = 1–10% of the fibre area; 2 + = 11–25%; 3 + = 26–50%; 4 + = 51–100%.

Computer image analysis
For stainings that featured a large number of positive vessels or large positive areas, computer image analysis was used to quantify the stained areas. IL-1α and HLA-DQ staining was analysed by image analysis. The images were assessed in a Quantimet 600 (Q600) image analyser (Leica Cambridge, Cambridge, UK). The image processor was controlled by a computer (PC). Special software was written for this application, in a high-level language (QUIPS). The results of the image analysis showed the positively stained area as a percentage of the total tissue section area. Image analysis was also used to assess the total biopsy specimen area in mm². Mean values of three measured sections from each biopsy were used.

Circulating cytokines, adhesion molecules and rheumatoid factors
Blood was drawn immediately after the muscle biopsy was taken. Serum was stored at −20°C and was available from seven ExRA patients and nine RA controls for the analysis of circulating cytokines, soluble adhesion molecules and RF isotypes, which was performed by a blinded investigator (LT). The ExRA patients subject to serum analysis suffered from pleuritis (n = 2), cutaneous vasculitis (n = 3), pericarditis (n = 1) and Felty’s syndrome (n = 1). Tumour necrosis factor α (TNF-α) was measured with an automated immunoassay system, Immulite (DPC, Los Angeles, CA, USA). sICAM-1, soluble VCAM-1 (sVCAM-1), soluble E-selectin and soluble L-selectin were measured by enzyme-linked immunoabsorbent assay (ELISA) using kits from R&D Systems (Minneapolis, MN, USA). IgM,
IgG and IgA RF isotypes were analysed by ELISA as described previously [27], which was calibrated against the WHO RF reference preparation.

Statistical analysis
Fisher’s exact test was used to assess differences between the ExRA and non-ExRA groups regarding previous RF and antinuclear antibody status and current treatment with DMARDs and corticosteroids. In comparisons of other clinical parameters and the results of serum analysis and immunohistochemistry studies, the paired t-test was used for parameters with a normal distribution (i.e. pairwise differences between cases and controls in areas positive for IL-1α by computer image analysis and pairwise differences for IgM RF titres). The results are presented as mean pairwise differences and 95% confidence intervals. For parameters without a normal distribution, the Wilcoxon signed rank test was used, and the results are presented as median pairwise differences and P values for between-group differences. Correlations between clinical, serological and immunohistochemical variables were investigated using Spearman’s correlation test.

Results
Histopathological assessment
In haematoxylin/eosin-stained sections, single, small, perivascular, cellular infiltrates were noted in one ExRA patient and one non-ExRA patient. There were, however, no inflammatory cell infiltrates surrounding or invading the muscle fibres and no signs of fibre degeneration. Specific staining showed scattered cells featuring CD3, CD4, CD8, CD68/163, CD19/20, LFA-1α and VLA-4, without differences between the groups.

Interleukin-1α and adhesion molecules in muscle tissue
IL-1α was expressed in endothelial cells of arterioles, venules and capillaries. The patients with ExRA had more extensive staining for IL-1α, especially in the arterioles (Fig. 1). The total area stained for IL-1α (by computer image analysis) was greater in ExRA patients than in non-ExRA controls with RA [mean pairwise difference 0.26%, 95% confidence interval (95% CI): 0–0.52]. The area stained in non-ExRA patients did not differ substantially from that in the healthy controls (Fig. 3). There was no difference in IL-1α expression between patients on glucocorticosteroid treatment and non-glucocorticoid-treated patients (P = 1.0). The size of the positive area correlated with the number of swollen joints [Spearman’s correlation coefficient (r) = 0.51; P = 0.03] in the total group studied, but not in the ExRA (r = 0.14; P = 0.71) or non-ExRA group (r = 0.42; P = 0.23) when studied separately. There was no correlation between IL-1α expression and CRP or Ritchie’s index.

ICAM-1 and VCAM-1 expression in endothelium and infiltrating cells was limited, without major differences between the groups (Table 1).

MHC class I and II expression
HLA-ABC and -DR antigens were expressed in the endothelium of most blood vessels in ExRA patients, non-ExRA patients and healthy controls, without obvious differences between the groups. Expression of HLA-DQ, on the contrary, was highly variable, from being almost absent in some samples to being present in hundreds of capillaries in some. The endothelium of the capillaries of some extensively stained sections from ExRA patients had a thickened appearance when stained for HLA-DQ (Fig. 2a), resembling the high endothelial venules of lymph nodes. The number of stained capillaries (by manual scoring) was significantly increased in ExRA patients (median 22.5 mm², IQR 15.9–74.6 vs 7.9, 5.9–15.5) (P = 0.039). There was also a trend towards a larger HLA-DQ-positive tissue area, as assessed by computer image analysis, in ExRA patients (median 0.28%, IQR 0.086–1.03 vs 0.10, 0.058–0.12) (P = 0.074) than in non-ExRA patients. There was a correlation between the number of HLA-DQ-positive capillaries and the total DQ-positive area in the total group studied (r = 0.75, P = 0.0003) as well as in the ExRA and non-ExRA subgroups separately. The HLA-DQ staining pattern in non-ExRA patients did not differ from that in the healthy controls (Fig. 4). There was no correlation between HLA-DQ expression and disease activity parameters (number of swollen joints, Ritchie’s index and CRP) in RA patients; this applied to the analysis of all the RA patients together and of the ExRA and non-ExRA groups studied separately.

HLA-ABC expression in muscle fibres was detected in five ExRA patients, five non-ExRA RA patients and one healthy control. In five sections, more than 10% of the muscle fibres (by visual scoring) were HLA-ABC-positive. Four of these had ExRA (one with Felty’s syndrome, two with pericarditis and one with cutaneous vasculitis) and one was a control RA patient.

Muscle fibres stained for HLA-DR were present in two ExRA patients, both of whom had presented with pericarditis. A relatively small number of fibres (<10%) were stained in both cases.

Serum analysis
Serum concentrations of TNF-α, sICAM-1, sVCAM-1 and IgM RF were significantly higher in the ExRA group (Table 2). The concentrations of TNF-α and sICAM-1 were highly correlated (r = 0.68, P = 0.003). TNF-α and sICAM-1 correlated with the number of swollen joints when all the RA patients were analysed as one group, but not in the two groups when studied separately. IgM RF levels were higher in the ExRA group (mean pairwise difference 938 IU/ml, 95% CI 182–1694), and IgM RF levels correlated with the number of HLA DQ-positive capillaries (r = 0.56, P = 0.02).
Discussion

In this systematic immunohistochemical study of muscle tissue, the expression of HLA-DQ and IL-1α was increased in the endothelium of blood vessels in skeletal muscle tissue of RA patients with ExRA compared with those without extra-articular manifestations. This occurred in the absence of clinical and histopathological signs of localized inflammation in the muscle tissue studied, indicating that the abnormal IL-1α and HLA-DQ expression may be a general feature of the vascular system of ExRA patients. Elevated serum levels of sICAM-1, sVCAM-1 and TNF-α were also found in ExRA patients compared with the non-ExRA controls. Concentrations of RF, in particular IgM RF, were also increased in ExRA. Moreover, IgM RF levels correlated with the number of capillaries expressing HLA-DQ in muscle tissue.

The ExRA cases included in this study manifested a spectrum of different systemic features of RA. They fulfilled the Malmö criteria for extra-articular disease, which have been demonstrated to identify patient groups with an increased mortality rate, especially in vascular disease, compared to RA patients in general [3, 11]. The present findings may thus be of relevance to the comorbidity and mortality associated with extra-articular RA.

The immunohistochemical protocols used in this study have previously been used extensively in studies of inflammatory myopathies [18, 26, 28]. We used computer image analysis to assess the staining of relatively small numbers of large areas (as in the case of the arteriolar staining for IL-1α), as the total area stained was considered to reflect the amount of cytokines produced rather than the number of positive
arterioles. We relied on conventional microscopy when the stained areas (cells and microvessels) were small and the visual counting of numbers instead of the percentage stained area was considered to be more accurate (e.g. when staining for lymphocyte and macrophage CD markers, adhesion molecules). In the case of HLA-DQ expression, for which the biopsies varied both in the number of stained vessels and in the area stained in each vessel, both methods were used, and we demonstrated a correlation between the number of stained capillaries (assessed by conventional microscopy) and the stained area (measured by image analysis).

The increased serum concentrations of TNF-α and sICAM-1 in the ExRA group, which did not correlate with the immunohistochemical findings, illustrate that several different inflammatory pathways may be involved in systemic RA. TNF-α is recognized as an important factor in the pathogenesis of RA [29]. Treatment with TNF-blocking agents reduces the serum level of sICAM-1 [30] and decreases clinical and laboratory signs of disease activity [31, 32], although the specific effect on extra-articular manifestations has not been evaluated.

In a recent study of rheumatoid vasculitis, increased expression of the adhesion molecules ICAM-1 and VCAM-1 and of HLA-DR was observed in muscle biopsy specimens from vasculitis patients with and without perivascular infiltrates, compared with controls with non-vasculitic RA and osteoarthritis [22]. Expression of HLA-DQ and IL-1α was, however, not evaluated in that study. As the case definitions were different from the criteria for ExRA used in the present series, the results are not directly comparable.

The staining patterns observed in microvessels of muscle tissue in the present study resemble the findings

![FIG. 3. Increased IL-1α staining shown by computer image analysis in ExRA patients compared with non-ExRA patients (mean pairwise difference 0.26%, 95% CI 0–0.52).](image)

![FIG. 4. Increased number of HLA-DQ-expressing capillaries in ExRA patients compared with non-ExRA patients (P = 0.039).](image)

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<th>TABLE 1. Stained vessels quantified by conventional microscopy</th>
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<td>Specificity</td>
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<td>HLA-DQ</td>
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<td>ICAM-1</td>
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NS = not significant.

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<th>TABLE 2. Circulating factors determined by ELISA</th>
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<td>Specificity</td>
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<td>TNF (ng/l)</td>
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described previously in endothelial cells of small blood vessels in the inflamed synovium of RA patients, where increased expression of IL-1α [33] and HLA-DQ [34, 35] was reported. On the contrary, synovial HLA-DQ expression in endothelial cells was not found in a group of patients with reactive arthritis [35], suggesting that this finding is specific for microvessels of RA patients.

Increased endothelial IL-1α staining in muscle tissue has also been found in patients with inflammatory myopathies [18, 26], but in the myositis patients IL-1α was expressed in the capillaries as well as in the arterioles, whereas in the present series the staining was more prominent in the arterioles. HLA-DQ expression in the endothelial cells of myositis patients was less prominent than in ExRA patients (unpublished data, own observations). This pattern may thus be characteristic of the rheumatoid disease process, and disseminated endothelial IL-1α and HLA-DQ expression could be important in the pathogenesis of ExRA. Felty’s syndrome and rheumatoid vasculitis have been reported to be associated with the genetic subtype HLA-DQ7 [36], and in a series of ExRA patients with a range of manifestations similar to those in the present patients, a trend towards an association with HLA-DQ7 was noted [37]. In the present series, there was a correlation between IgM RF levels and the number of HLA-DQ-positive capillaries in muscle tissue. A possible mechanism could be the induction of endothelial HLA-DQ expression by circulating immune complexes in genetically predisposed individuals.

The major limitations of the present study are the limited number of patients in each group, the possibility of significant findings due to multiple testing, and the possible influence of factors not controlled for in the analysis. The results should therefore be confirmed in a larger study. The distribution of ExRA manifestations was similar to that found in a retrospective survey of ExRA from a well-defined RA population [3], indicating that the ExRA sample is representative of this subgroup of RA patients. The matching of control RA patients from the same catchment area for age, sex and disease duration should prevent selection bias among the controls as well as confounding by the potential risk factors for vascular disease. The non-ExRA patients all had non-nodular disease, as rheumatoid nodules are known predictors of severe extra-articular disease [37]. There was a trend towards fewer swollen joints in the non-ExRA patients at inclusion, but there was no correlation between joint count indices or CRP on one side and the expression of IL-1α or HLA-DQ on the other side, in either the ExRA or the non-ExRA group. It is, however, still possible that the increased HLA-DQ and IL-1α expression in the ExRA group may in part be explained by more widespread articular inflammation. The relative importance of this could be elucidated by stratification by the number of swollen joints in a larger series, but such an analysis was not possible for the present series. However, the vascular staining pattern described may be an important pathophysiological mechanism in systemic RA, regardless of whether it is determined in part by the extent of articular inflammation.

Endothelial activation has been defined as ‘a quantitative change in the level of expression of specific gene products (i.e. proteins), which in turn endow endothelial cells with new capabilities that cumulatively allow endothelial cells to perform new functions’ [38]. IL-1α expression has been reported in arteriosclerotic lesions, but not in normal blood vessels [39]. IL-1α induces tissue factor-like procoagulant activity and plasminogen activation inhibitor synthesis in human endothelial cells in vitro [40], indicating that increased IL-1α production may have a procoagulant effect on vascular endothelium. MHC class II expression occurs in endothelial cells in some, but not all large blood vessels [41], and has been found in endothelium overlying atherosclerotic plaques [42]. IL-1α and HLA-DQ may thus be important in atherosclerotic vascular disease. Endothelial activation of this sort could be important in other systemic inflammatory conditions, although this requires further study.

In conclusion, we have demonstrated increased expression of HLA-DQ and IL-1α in the endothelial cells of skeletal muscle blood vessels in patients with extra-articular manifestations of RA compared with non-extra-articular RA controls. This may indicate systemic endothelial activation in extra-articular RA. Serum levels of IgM RF, TNF-α and circulating sICAM-1 and sVCAM-1 were also increased in extra-articular RA compared with non-ExRA patients, indicating that several inflammatory pathways may be involved in extra-articular disease. Further studies should be performed in order to investigate possible associations between the immunohistochemical findings, individual extra-articular manifestations and other clinical features, including cardiovascular disease.

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