Modification of Pro- and Antiinflammatory Cytokines and Vascular-Related Molecules by Tumor Necrosis Factor-α Blockade in Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Analysis of serum concentrations and modifications of tumor necrosis factor-α (TNF-α), its soluble receptors (TNFR), interleukin 10 (IL-10), and vascular related molecules [soluble vascular cell adhesion molecule 1 (sVCAM-1), vascular endothelial growth factor (VEGF)] after therapy with methotrexate (MTX) and anti-TNF (infliximab) in patients with rheumatoid arthritis (RA).

Methods. Thirty-six patients with RA and 20 healthy controls were included. Patients had been orally taking a stable dose of MTX of at least 12.5 mg/week for a minimum of 6 months before inclusion in the study. Twenty-five patients had shown a clinical response to MTX (MTX Group). The other 11 had shown an unsatisfactory response and presented with active RA; they were selected for additional treatment with infliximab (MTX + IFM Group). Disease activity score (DAS28), hemoglobin concentration, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and serum levels of TNF-α, soluble TNFR, IL-10, sVCAM-1 and VEGF were determined at baseline and prior to every infusion of infliximab (3 mg/kg) at 2, 6, 14, 22, and 30 weeks.

Results. Although serum levels of TNF-α were similar in patients and controls, patients showed significantly higher concentrations of both soluble TNFR (sTNFR55 and sTNFR75), IL-10, sVCAM-1, and VEGF than healthy individuals. Significantly higher levels of sVCAM-1 and VEGF, but not of the other tested molecules, were detected in those with active disease. After infliximab treatment (MTX + IFM Group) there was a significant decrease in DAS28 and modified Health Assessment Questionnaire scores and ESR and CRP levels. Serum concentration of VEGF showed a significant decrease after infliximab, with levels comparable to those of patients with inactive RA, although VEGF continued to present higher values than in healthy controls.

Conclusion. Increased levels of vascular related molecules sVCAM-1 and VEGF are serum markers of active RA. The absence of normalization of levels of these molecules in patients with inactive RA could be one of the reasons response to therapy is only temporary. (J Rheumatol 2005;32:2102-8)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
ANTI-TUMOR NECROSIS FACTOR-α
TUMOR NECROSIS FACTOR-α
VASCULAR ENDOTHELIAL GROWTH FACTOR 1
VASCULAR CELL ADHESION MOLECULE 1
INTERLEUKIN 10

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by inflammation of the synovial joints, infiltration by blood derived cells, and eventual destruction of cartilage and bone, as well as extraarticular symptoms and signs. It is generally accepted that the activity of the cells within the synovium and, in particular, the cytokine and enzyme products they generate, are involved in the destruction of the underlying matrix components[1].

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A wide range of cytokines and other inflammatory mediators are expressed in these joints in RA. However, the arguments that place tumor necrosis factor-α (TNF-α) at the heart of the inflammatory process in RA are particularly compelling[2]. TNF-α and its 2 receptors (p55 and p75 TNFR) are expressed at several sites within the synovial membrane, including the cartilage-pannus junction[3]; tissue expression of these molecules is reflected in synovial fluid and in serum, where elevated concentrations of TNF-α and soluble forms of the receptors (sTNFR) are detected[4,5]. These soluble forms of receptor represent shed cell-surface receptors, which may function as natural regulators of TNF-α activity[5]. Clinical investigations in which the activity of TNF-α in patients with RA was blocked with intravenously administered infliximab, a chimeric anti-TNF-α monoclonal antibody, have shown that TNF-α regulates the inflam-

matory process, controls symptoms, and retards joint damage[6-9].

Among the effects of proinflammatory cytokines such as TNF-α, the expression of adhesion molecules on the endothelial surface must be considered[10]. Inflammatory cells migrate into the joints by a multistage process that involves an initial loose-attachment stage followed by firm adhesion. Firm adhesion is mediated by binding of integrin molecules, present in inflammatory cells, and immunoglobulin gene superfamily proteins, present on activated endothelial cells. Vascular cell adhesion molecule-1 (VCAM-1, CD106) is a representative molecule of the latter group[10]. Immunoglobulin gene superfamily molecules are susceptible to proteolytic cleavage and may also exist in the circulation in soluble form (sVCAM-1)[11]. Increased expression of VCAM-1 and of other VCAM has been documented in the synovium of patients with RA[12].

In addition, the role of pannus formation in RA must be stressed. The established phase of RA is associated with an expansion of synovial tissue due to proliferation and infiltration of cells of lymphohematopoietic origin. Formation of new microvessels from the preexisting vasculature — a process known as angiogenesis — is essential in maintaining and nourishing synovial tissue mass[13,14]. Vascular endothelial growth factor (VEGF) is a potent angiogenic molecule that promotes migration and proliferation of endothelial cells[15]. Synovial fluid and serum levels of VEGF in patients with RA are increased[16,17]. Interestingly, TNF-α and IL-6 upregulate VEGF production in RA[15,18].

The extent of the inflammatory response is partly controlled by antiinflammatory compounds, such as interleukin 10 (IL-10)[19]. IL-10 may suppress TNF-α activity by an inhibition of TNF-α secretion and downregulation of the expression of surface TNFR[20,21]. Although IL-10 expression is upregulated and hence relatively abundant in RA synovium[22], the effect of treatment of RA on IL-10 has been studied only in a small group of patients[23].

We tested the hypothesis that the treatment induced clinical response of RA patients tends to normalize the altered pattern of cytokines (both pro- and antiinflammatory) and vascular factors detected in them. Thus, we analyzed serum concentrations and modifications of TNF-α, soluble TNFR, IL-10, and vascular related molecules (sVCAM-1, VEGF) after therapy in RA patients distributed into 2 groups according to response to methotrexate (MTX)[24]: those patients not responding to MTX were additionally treated with anti-TNF-α.

MATERIALS AND METHODS

Thirty-six patients with RA according to the criteria of the American Rheumatism Association[25] and 20 healthy controls were studied. Patients were selected consecutively from those attending the Rheumatology Unit of the Hospital Universitario Puerta del Mar, Cadiz, Spain. They had been taking a stable dose of MTX orally, at least 12.5 mg/week, for a minimum of 6 months before entering the study. Twenty-five patients had shown a clinical response (MTX Group). The other 11 had shown an unsatisfactory response to this disease modifying drug and presented with active RA [≥ 6 swollen or tender joints, and ≥ 2 of the following: morning stiffness that lasted at least 45 min, erythrocyte sedimentation rate (ESR) ≥ 30 mm/h, and serum C-reactive protein (CRP) concentration ≥ 2 mg/dl]; they were selected for additional treatment with infliximab (Remicade; Centocor, Malvern, PA, USA, and distributed by Schering Plough, Canada Inc.) (MTX + IFM Group). Patients were permitted to continue taking low dose oral corticosteroids (prednisone < 10 mg/day) and/or nonsteroidal antiinflammatory drugs at a stable dose.

Patients with RA were excluded if they had any of the following: (1) clinical evidence of infection; (2) neoplasia; (3) cardiovascular disease (stroke, intermittent claudication, ischemic cardiopathy, heart failure), attributed to atherosclerosis on the level of endothelial adhesion molecules; (4) gastrointestinal bleeding or shock; (5) red blood cell or plasma transfusion in the month prior to inclusion in the study (to rule out possible interference of contamination or reactions between these elements and the inflammatory and immune systems); (6) pregnancy; and (7) hypersensitivity to MTX, infliximab, or to the inactive ingredients of the drugs.

Sex and age matched healthy individuals, selected from hospital workers, constituted the control group; absence of evidence of infection was confirmed by clinical history, physical examination, and laboratory tests (no increase in leukocyte count, normal values of ESR and CRP, and normal urinalysis). Informed consent was obtained from all patients and controls. The protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and had been approved by the institutional human research committee.

Study schedule and measurements. Patients were admitted to our research unit for participation in the study; a detailed history and physical examination was performed. The Disease Activity Score (DAS28) assesses disease activity including tender and swollen joint count, ESR, and general health status. It ranges from 0 (lowest) to 10 (highest activity)[26]. The criterion of clinical response used in the study was the definition of the European League of Associations Against Rheumatism (EULAR)[27]: DAS28 index had decreased by more than 1.2 points with reference to baseline.

The Modified Health Assessment Questionnaire (MHAQ) completed by the patient evaluates physical functional impairment. Scores range from 0 (no impairment) to 3 (maximum impairment)[28]. The Spanish version of the MHAQ has previously been validated[29].

After overnight fasting, hemogram and leukocyte count, ESR, elemental serum biochemistry (electrolytes, urea, creatinine; standard liver function tests; transaminases; bilirubin, albumin), rheumatoid factor (RF), and CRP were determined. Blood samples collected in sterile Vacutainer tubes were centrifuged (3500 rpm for 15 min at 4°C) and serum stored at –80°C in pyrogen-free polyethylene tubes until TNF-α, soluble TNFR, IL-10, sVCAM-1, and VEGF were assayed.

Patients of the MTX + IFM Group were treated with infliximab (3 mg/kg at Weeks 0, 2, 6, 14, 22, and 30). DAS28 and MHAQ were determined and blood samples for cytokines, cytokine receptors, and vascular related molecules were obtained before each infusion of infliximab.

Laboratory determinations. Hemograms, ESR, elemental serum biochemistry, RF, and CRP were measured by standard laboratory methods. Serum levels of TNF-α, sTNF receptors, IL-10, sVCAM-1, and VEGF were assayed with ELISA kits (R&D, Minneapolis, MN, USA) according to manufacturer's instructions, with the following detection limits (lowest positive standard): TNF-α 4.4 pg/ml; TNFR p55 3.0 pg/ml; TNFR p75 1.0 pg/ml; IL-10 3.9 pg/ml; sVCAM-1 2 ng/ml; and VEGF 9 pg/ml.

Statistical analysis. Data are presented as median and interquartile range or, when indicated, as absolute number and percentage. The data from 2 independent groups were compared with the Mann-Whitney test. The significance of parameters within each group was tested by the Wilcoxon matched-pairs signed-rank test. For qualitative variables, chi-square with Yates' correction or Fisher's exact test was used. Correlations were

assessed by Spearman's method. A p value < 0.05 was considered significant. Statistical analysis was performed using the SSPS 11.0 program.

RESULTS

Baseline clinical characteristics. At baseline, there were 25 patients with response to MTX (MTX Group) and 11 nonresponders (MTX + IFM Group). Baseline characteristics of both groups, prior to infusion of infliximab in the MTX + IFM group, are presented in Table 1. Higher DAS28 and MHAQ scores, ESR and CRP levels, and a lower hemoglobin concentration were detected in the MTX + IFM group.

Baseline concentrations of cytokines, cytokine receptors, and vascular related molecules. Although serum levels of TNF-α were similar in patients and controls, patients showed significantly higher concentrations of both soluble TNFR, sTNFR55 and sTNFR75, and of IL-10 than healthy individuals. Similarly, serum concentrations of the vascular related molecules sVCAM-1 and VEGF were significantly higher in RA patients than in controls (Table 2). Moreover, compared with healthy controls, even in patients with a DAS28 index < 2.6, we detected significantly higher serum levels of both the soluble TNFR, IL-10, sVCAM-1, and VEGF (data not shown).

Concentrations of TNF-α, soluble TNFR, and IL-10 were similar in patients with active RA (MTX + IFM Group) and in those with MTX induced inactive disease (MTX Group). However, significantly higher levels of sVCAM-1 and VEGF were detected in those with active disease (Table 2).

A significant correlation was detected between VEGF levels and platelet counts in the group of all patients ($r = 0.448$, $p = 0.006$). Nevertheless, no significant correlation was detected between cytokines, cytokine receptors, and vascular related molecules and activity scores, ESR, or CRP concentration ($p > 0.05$ in each case).

Modification of clinical and laboratory indicators after infliximab. Patients with active disease after MTX treatment were treated with infliximab. There was a significant clinical improvement of RA during this therapy as judged by a

Table 1. Baseline characteristics of patients with RA. Except when indicated, data are median (interquartile range).

Characteristic	MTX Group	MTX + IFM Group	p
No. of patients	25	11	
Age, yrs	57 (48–64)	48 (36–58)	0.03
Female sex, n (%)	19 (76.0)	9 (81.2)	0.50
Rheumatoid factor positive, n (%)	19 (76.0)	8 (72.7)	0.50
Disease duration, yrs	7 (2–21)	7 (2–13)	0.90
Duration of therapy with MTX, yrs	3 (1–6)	2 (1–2)	0.06
DAS28 score	2.6 (2.2–3.2)	6.6 (6.3–7.2)	< 0.001
MHAQ score	0 (0.0–0.7)	1.9 (1.7–2.0)	< 0.001
Hemoglobin concentration, g/dl	12.5 (11.0–14.2)	10.8 (9.2–12.0)	0.009
Platelet counts, cells/mm^3 × 1000	342 (254–436)	430 (325–455)	0.23
ESR, mm/h	34 (25–55)	75 (60–95)	< 0.001
CRP, mg/dl	0.8 (0.5–1.5)	4.3 (3.5–5.4)	< 0.001

MTX group: Patients with clinical response to MTX (inactive RA). MTX + IFM group: Patients without clinical response to MTX (active RA) who are then also treated with infliximab. NS: nonsignificant.

Table 2. Baseline concentrations of cytokines, cytokine receptors, and vascular related molecules in healthy controls and patients with RA, grouped by response to MTX.

	Healthy Controls (n = 20)	Patients with RA	
		MTX group (n = 25)	MTX + IFM group (n = 11)
TNF-α, pg/ml	0.6 (0.0–0.9)	0.1 (0.0–1.8)	0.6 (0.0–1.4)
sTNFR55, pg/ml	2.0 (1.0–5.9)	155.4 (114.3–182.9)**	156.1 (136.6–190.7)**
sTNFR75, pg/ml	6.3 (3.4–11.0)	211.2 (184.4–267.5)**	191.7 (178.4–266.4)**
IL-10, pg/ml	0.6 (0.0–1.3)	6.5 (5.1–10.1)*	4.7 (4.4–8.2)*
sVCAM-1, ng/ml	570 (433–684)	1110 (904–1281)**	1584 (1223–1875)**‡
VEGF, pg/ml	212 (98–504)	637 (326–1053)**	1064 (616–1557)**†

* $p < 0.05$ vs healthy controls; ** $p < 0.001$ vs healthy controls; ‡ $p < 0.05$ vs group MTX; † $p < 0.01$ vs group MTX. MTX group: Patients with clinical response to MTX (inactive RA). MTX + IFM group: Patients without clinical response to MTX (active RA) who are then also treated with infliximab. Tumor necrosis factor alpha (TNFα), its soluble receptors p55 and p75 (sTNF55 and sTNF75), interleukin 10 (IL-10), soluble vascular cell adhesion molecule 1 (sVCAM-1), and vascular growth endothelial factor (VEGF)..

decrease in the DAS28 and MHAQ scores. Similarly, a significant increase of hemoglobin concentration and decrease of ESR and CRP concentration was detected (Figure 1).

Serum concentrations of TNF-α, but not of soluble TNFR, increased significantly during infliximab treatment. In contrast, concentrations of IL-10 and sVCAM-1 showed nonsignificant changes. Concentrations of VEGF showed a significant decrease after infliximab (Figure 2). However, even at the end of the followup, levels of this molecule continued to be significantly higher than in the healthy controls [RA patients 632 (range 480–742) pg/ml; healthy controls 212 (range 98–504) pg/ml; p = 0.022].

DISCUSSION

RA is a chronic systemic disease characterized by an inflammatory erosive synovitis[1,2]. Several treatments have been proposed, and currently MTX is the preferred disease modifying drug. It has been proved that MTX induces downregulation of monocyte activation in RA: the synthesis and concentrations of monokines such as TNF-α and IL-1ß in synovial membrane, synovial fluid, and peripheral blood are reduced. Further, the concentration of IL-6 decreases in responders to MTX therapy[30]. Finally, synthesis of antiinflammatory IL-10 increases during the treatment[23].

Our study did not analyze a cohort of patients with RA longitudinally from the beginning until remission, but we did determine the clinical characteristics and concentrations of proinflammatory (TNF-α) and antiinflammatory (soluble TNFR, IL-10) molecules, as well as those implicated in the endothelial adhesion (sVCAM-1) or angiogenesis (VEGF), as determined by clinical response (clinically inactive RA), or its absence (clinically active RA). Increased concentrations of soluble TNFR, molecules capable of binding TNF-α and hence acting as inhibitors by competing with mem-

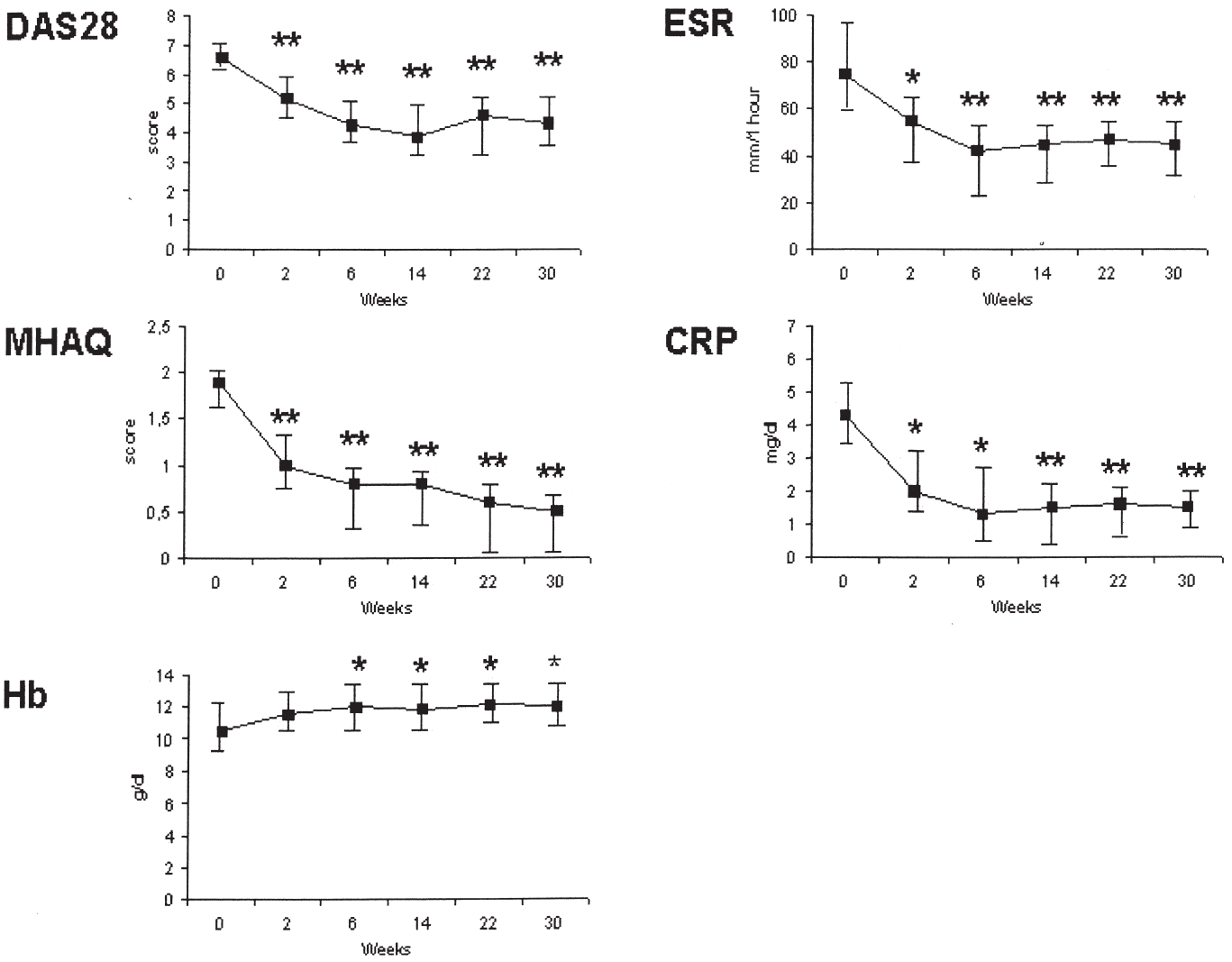

Figure 1. Evolution of clinical and laboratory measures in patients with RA after treatment with MTX and infliximab. Results are shown as median (interquartile range). *p < 0.05 vs baseline. **p < 0.001 vs baseline. Hb: hemoglobin.

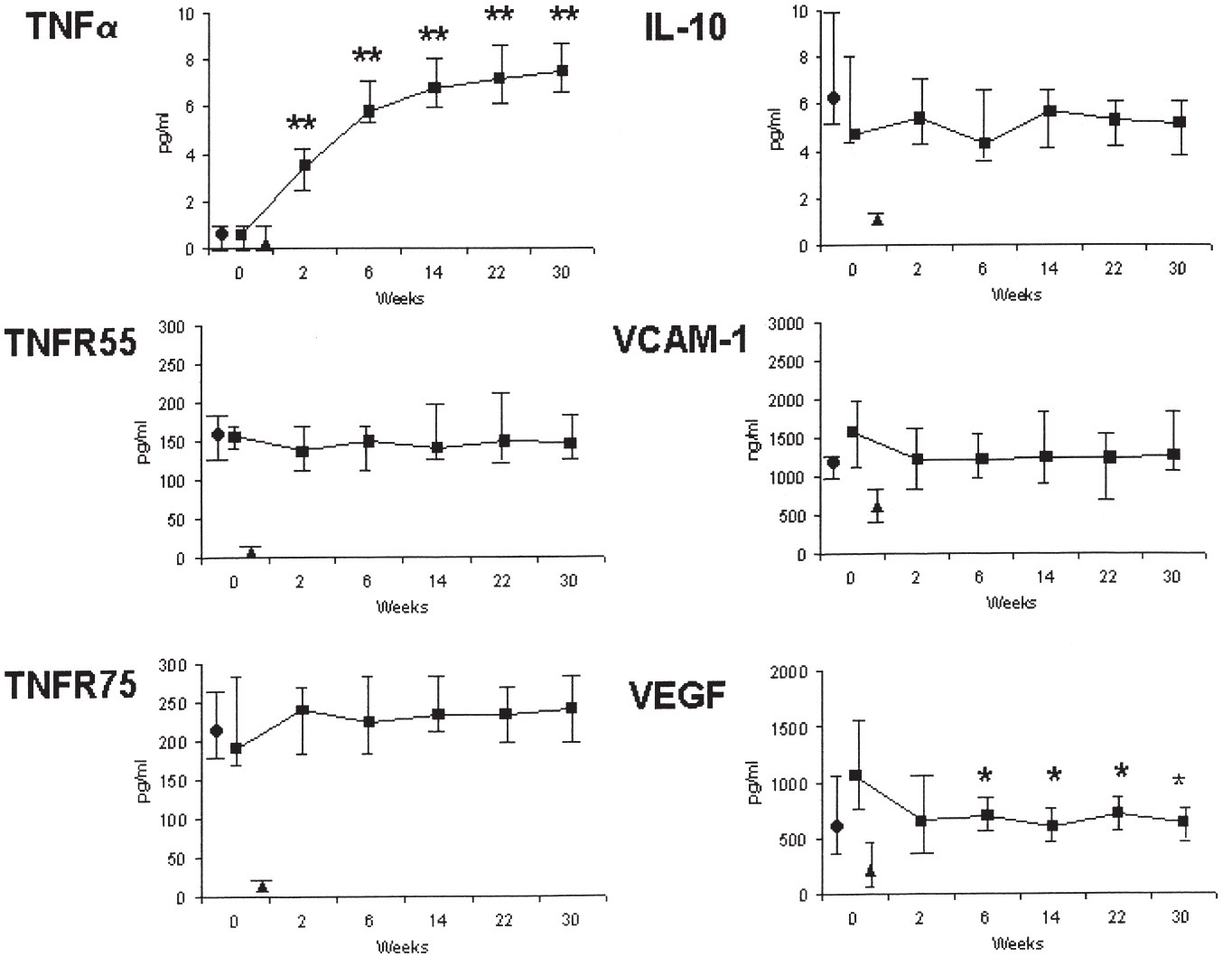

Figure 2. Evolution of serum concentrations of TNF-α, soluble TNF receptors TNFR55 and TNFR75, IL-10, VCAM-1, and VEGF in patients with RA after treatment with MTX and infliximab. Results are shown as median (interquartile range). *p < 0.05 vs baseline. **p < 0.001 vs baseline. For comparison, values in healthy controls (s) and the MTX Group (◨) are shown in Week 0.

brane-bound signaling receptors[1,2,5], and antiinflammatory cytokines (IL-10) were observed in patients with inactive RA, in comparison with healthy controls. However, even in these patients with inactive disease, increased levels of vascular related molecules implicated in adhesion and angiogenesis persisted. These elevated concentrations of sVCAM-1 and VEGF have been detected in patients with RA prior to treatment[1]. This implies the maintenance of inflammatory alterations, although at a subclinical level, after a median of 3 years of MTX therapy.

Patients with active RA differ from those responding to MTX in having higher serum levels of VCAM-1 and VEGF. It is possible to hypothesize that the lower concentration of these molecules (and of the pathological processes they represent: inflammatory infiltration and formation of pannus) in patients with inactive RA could be an effect of MTX treatment. Other studies have detected a significant correlation between synovial fluid and/or serum levels of VEGF and ESR, serum concentration of CRP, or number of articulations with active inflammation, suggesting a relationship between VEGF and disease activity indices[16,31]; this relationship was not corroborated in other studies[32,33]. Studies that described the correlation were performed in patients with a short period of disease evolution, but not in those with more than 5 years of followup.

The clear outcomes of infliximab therapy[6] provide an opportunity to probe deeper into the role of TNF-α in the pathogenesis of RA, and into the role of other cytokines and vascular endothelial molecules associated with leukocyte migration or angiogenesis in these patients. Infliximab therapy was clinically useful in our patients[6], and induced an improvement of the routine markers of inflammatory activity (elevated ESR and CRP or decreased hemoglobin concentration). It has been proved that anti-TNF-α downregu-

lates several proinflammatory cytokines (IL-1, TNF-α, IL-6) and chemokines (IL-8, monocyte chemoattractant protein-1)[7,8]. Modifications of some of these indicators were also evident in our patients. Thus, increase of TNF-α was observed in patients after anti-TNF-α treatment. This finding has been attributed to the formation of biologically inactive TNF-α/anti-TNF-α complexes[7]. However, new increases of the concentration of antiinflammatory compounds, soluble TNFR, and IL-10 were not detected. This implies that these molecules are probably not responsible for the clinical results of infliximab therapy.

TNF-α induces the expression of endothelial adhesion molecules, and infliximab therapy reduces the expression of VCAM-1 in the synovium of RA patients[12]. A more quantitative measure of adhesive properties of blood vessels is the serum sVCAM-1 concentration. Serum sVCAM concentration was reduced after the first infliximab infusion, although differences compared to baseline values were not significant, suggesting the potential persistence of the ability of circulating inflammatory cells to infiltrate the affected joints. Although this is controversial[34], the absence of significant changes of serum VCAM-1 concentration after 2 weeks of infliximab has been observed previously[35]. Our data are in agreement with these findings and contribute to the knowledge of these processes, showing that the serum concentration of VCAM-1 did not change significantly even though infliximab was administered for a relatively long period (30 weeks).

TNF-α regulates the expression of VEGF in the synovium of RA patients[17,18]. VEGF is an endothelial cell-selective angiogenic factor with a leading role in the angiogenic process of RA[13]. Angiogenesis is considered to be central in the delivery of cells and molecules to the developing RA lesion and in the maintenance of the increased mass of pannus, which will invade cartilage and bone, leading to irreversible damage and eventual joint failure[15]. Indeed, the increase of VEGF concentrations has been associated with erosive arthritis, in contrast to self-limited arthritis[36]. Interestingly, a significant decrease of VEGF was evident after infliximab treatment. This would be associated with diminished clinical features of arthritis; and thus a decrease in DAS28 score and CRP concentration mirrored the reduction of this angiogenesis marker. Further, this finding probably contributes to the ability of the combined MTX plus infliximab treatment to halt the progression of erosive changes and joint space narrowing[37].

Remission is rare in late-stage active RA[38]. The persistence of significant increases of sVCAM and VEGF in comparison with healthy controls could be one factor explaining why the response to MTX or infliximab in RA patients is only temporary, with relapse of the arthritis occurring in a high percentage of patients after the suspension of the therapy[39]. These pathways, likely to be involved in maintaining active disease, could also be important targets for therapy.

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