EXTENDED REPORTS

Monocyte chemoattractant protein 1 (MCP-1) in temporal arteritis and polymyalgia rheumatica

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Abstract

Objective—To examine the localisation of monocyte chemoattractant protein 1 (MCP-1) in the inflamed vessel wall in temporal arteritis (TA) and to measure MCP-1 in plasma both in patients with TA and patients with polymyalgia rheumatica (PMR).

Methods—By immunohistochemical techniques MCP-1 was localised to the vessel wall in patients with TA. In TA, PMR, and healthy controls MCP-1 was quantified by enzyme linked immunosorbent assay (ELISA) in plasma.

Results—MCP-1 was localised to the majority of mononuclear cells, some smooth muscle cells, and giant cells in the arterial biopsy specimens from 12 patients with histologically verified TA. In all sections, including the vasa vasorum, the endothelium stained positive. In the intima 73% (range 57–91%), in the media 49% (range 32–67%), and in the adventitia 74% (range of 62–91%) of all cells stained positive. In plasma MCP-1 was significantly raised in untreated TA (n=33) and untreated PMR (n=27) compared with healthy controls (n=12). Untreated TA plasma levels of MCP-1 (mean 391 pg/ml (range 82–778 pg/ml)) were similar to untreated PMR plasma levels (mean 402 pg/ml (range 29–1153 pg/ml)), and no significant difference was found between the two groups of patients. In both patients with TA and patients with PMR no correlation was found between the plasma level of MCP-1 and the erythrocyte sedimentation rate, haemoglobin concentration, and CD4/CD8 ratio.

Conclusions—These results show that MCP-1 plays a part in the disease processes of TA and PMR.

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Temporal arteritis (TA) and polymyalgia rheumatica (PMR) are both common diseases with similar clinical features.

It has been shown that erythrocyte sedimentation rate (ESR) and plasma levels of C reactive protein (CRP), interleukin 6 (IL6), soluble IL2 receptor (sIL2r), soluble CD8, and

peripheral CD4/CD8 are altered in patients with active disease.^{1–5}

The association between TA and PMR is a subject of continuing discussion. In both TA and PMR a reduction in CD8+ T lymphocytes in peripheral blood has been seen and correlated with disease activity.^{4–7} By polymerase chain reaction, Weyand *et al* verified that macrophage and T lymphocyte cytokines, including IL1 β , IL2, IL6, and interferon γ (IFN γ), are synthesised in the vessel wall in TA.⁸ The cytokine primer profiles in PMR were, however, less complete than for TA.⁸

The observation that monocyte chemoattractant protein (MCP-1) is a strong chemotactic factor for T lymphocytes and monocytes *in vitro*, and that it could be purified from various components of the vessel wall,^{9–10} led to the hypothesis that MCP-1 might be partly responsible for the recruitment of monocytes and T lymphocytes to inflammatory lesions in vessels.

Here we report that MCP-1 is localised in the arterial wall in TA and that plasma levels of MCP-1 are raised in patients with untreated TA and untreated PMR.

Material and methods

STUDY SUBJECTS

For immunohistochemistry, arterial wall sections were obtained from biopsy specimens of the temporal artery (also used for routine diagnostic microscopy) of 12 subjects with histologically verified TA and five subjects with either fever, weight loss, anaemia, or raised ESR of unknown origin. The histological criteria used for a positive biopsy were destruction of the internal lamina elastica membrane and infiltration by mononuclear cells. A pathologist verified the diagnosis. The 12 patients (four male, eight female) fulfilling the histological criteria had a mean age of 72.5 years (range 64–85). At the time of biopsy four were being treated with corticosteroids, three with non-steroidal anti-inflammatory drugs (NSAIDs), and five were receiving no anti-inflammatory treatment. Their mean ESR was 81 mm/1st h (range 50–132).

Table 1 lists the characteristics of the five patients who did not fulfil the criteria for TA or PMR. All 17 patients were recruited at the University Hospital of Århus.

For the quantification of plasma MCP-1 by enzyme linked immunosorbent assay (ELISA)

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Table 1 The distribution and number of monocyte chemoattractant protein 1 (MCP-1) positive cells throughout the arterial wall in temporal artery biopsy specimens from 12 patients with temporal arteritis (TA) (histologically verified) and five patients with negative biopsy and not fulfilling the criteria for polymyalgia rheumatica (PMR)

	Intima	Media	Adventitia	Endothelium
Biopsy positive TA (n = 12) (mean and range)*	73 (57–91)	49 (32–67)	74 (62–91)	Pos. in all sections
Biopsy negative (n=5) Sex/age/histology				
F/72/Inflamed adventitia. Treatment NSAID†, ESR† 86 mm/1st h	0	0	39	Neg.
M/80/Atherosclerosis. No treatment, ESR no inf	12	7	24	Weakly pos.
M/81/Atherosclerosis. No treatment, ESR 80 mm/1st h	29	30	65	Pos.
M/58/Atherosclerosis. Treatment prednisone, ESR 33 mm/1st h	8	5	45	Weakly pos.
M/79/Atherosclerosis. Treatment NSAID, ESR 59 mm/1st h	11	8	31	Weakly pos.

*Age mean 73 (range 64–85). At the time of the biopsy four patients were being treated with corticosteroids, three with NSAIDs, five were receiving no anti-inflammatory treatment. Mean ESR 81 mm/1st h (range 50–132).

†NSAID = non-steroidal anti-inflammatory drug; ESR = erythrocyte sedimentation rate.

and T lymphocyte subset determination in peripheral blood, 60 untreated subjects with TA/PMR were included in the study. All patients were recruited at the county hospital of Randers. A 3 cm long biopsy sample of the temporal artery was obtained in conjunction with routine biochemistry. Sampling of plasma and determination of T lymphocyte subset was performed before treatment with corticosteroids. The inclusion criteria were (1) persistent pain and stiffness in the shoulder or pelvic girdle, or both, worse in the morning; (2) clinical abnormality of a temporal artery; (3) fever, anaemia, weight loss, tiredness, and malaise; (4) a prompt and longlasting response to corticosteroid treatment, including normalisation of the ESR and haemoglobin (evaluated after one year). Criterion (4) and any two of the others were needed for inclusion. After one year of observation and treatment patients were divided into two groups: (a) patients with TA, who were defined by a positive biopsy, longlasting response to corticosteroid treatment, and no other known rheumatological disease and (b) patients with PMR, who were defined by having a negative biopsy, a longlasting response to corticosteroid treatment, and not fulfilling any other criteria for rheumatological diseases. All patients classified as PMR (biopsy negative) fulfilled criteria (1), (3), and not (2). The patients with a positive biopsy all presented with myalgia, except for five patients who fulfilled criteria (2) and (3).

The histological criteria used for a positive biopsy have been described already. The temporal artery biopsy sample was immediately fixed in formalin and then divided into four or five parts, embedded in paraffin, and serial sectioned (15 sections) for routine histological diagnosis (haematoxylin/eosin and elastin staining) by a pathologist.

Thirty three patients with a positive biopsy (TA), 25 female, eight male, had a mean age of 71.4 years (range 57–88). Twenty seven patients with a negative biopsy (PMR), 20

female, seven male, had a mean age of 67.3 years (range 52–84). Table 2 lists the biochemical characteristics of these two patient groups.

Plasma MCP-1 was also analysed in 12 healthy subjects, six male, six female, recruited among staff members, with a mean age of 43 years (range 33–59).

The study was carried out at the Department of Rheumatology, Århus University and Department of Medicine, Randers County Hospital, Denmark. It was approved by the local ethics committee of Århus County.

IMMUNOHISTOCHEMISTRY

From each of the biopsy specimens used for the histological diagnosis, 15 sections were used for haematoxylin/eosin and elastin staining, and three to five sections were used for immunohistochemical staining as follows.¹¹ Briefly, a rabbit was repeatedly injected with human recombinant MCP-1 (hrMCP-1). The IgG fraction was removed from the serum by $(\text{NH}_4)_2\text{SO}_4$ precipitation and thereafter dialysed in phosphate buffered saline (PBS). To the IgG fraction of biotinamidocaproate the *N*-hydroxysuccinimide ester (Sigma B-2643, St Louis, USA) was added in 50% DMSO, allowed to stand for two hours at room temperature and dialysed in a 100 kDa tube (Spectrum, Hudson, Texas, US) against 5 litres of PBS. Sections were deparaffinated, rehydrated through alcohol, permeabilised by boiling in 10 mM acetate buffer (pH 6.0) in a microwave oven for 3 × 5 minutes, and subsequently cooled in 10 mM Na_2CO_3 , NaHCO_3 (pH 9.0) for 30 minutes. Sections were blocked for 20 minutes in 10% v/w of normal rabbit serum (NRS) and Tris buffered saline (TBS, 50 mM, pH 7.4). Sections were incubated with the biotinylated polyclonal rabbit antibody, bio-Ra-anti-MCP-1 (1.25 mg/ml diluted 1:800), in 1% v/w NRS/TBS overnight at 4°C. The bio-Ra-anti-MCP-1 was detected by streptavidine-alkaline phosphatase (1:150, Amersham, UK). The staining was developed with fast red-TR salt (Sigma, St Louis, USA). Levamisole (Sigma, St Louis, USA) was added to inhibit the endogenous alkaline phosphatase. After five minutes' incubation the reaction was stopped in distilled water. Positive staining appears red.

Specific staining was verified by (a) substitution of the primary antibody with biotinylated normal rabbit IgG, (b) further dilution of primary antibody, and (c) preincubation of

Table 2 Monocyte chemoattractant protein 1 (MCP-1) levels in plasma, CD4/CD8 ratio, erythrocyte sedimentation rate (ESR), and haemoglobin in patients with untreated temporal arteritis (TA) and polymyalgia rheumatica (PMR)

	TA (n=33)			PMR (n=27)		
	Mean	Median	Range	Mean	Median	Range
Plasma MCP-1 (pg/ml)	391	342	82–778	402	356	29–1153
CD4/CD8	3.98	3.36	1.0–12.6	4.2	3.7	1.2–8.4
ESR (mm/1st h)	86	89	13–145	71	66	2–355
Haemoglobin (mmol/l)	6.7	6.6	5.1–8.0	7.4	7.3	5.0–9.7

bio-Ra-anti-MCP-1 with hrMCP-1 in a concentration of 10 mg/ml for 30 minutes at room temperature before staining. All resulted in negative staining.

Figure 1 Overview of a section of the temporal artery (magnification $\times 16$) showing differentiated mononuclear infiltration in relation to the lamina elastica externa, marked with arrows. Positive staining appears red, streptavidine-alkaline phosphatase.

Figure 2 (a) Section of the temporal artery (magnification $\times 80$) showing the distribution of MCP-1 positive cells located in the adventitia, media, and intima. Staining of the vasa vasorum in the adventitia is shown (point A). Mononuclear cell infiltration adjacent to the lamina elastica externa (point B). (b) Control staining of section from the same biopsy (substitution of the primary anti MCP-1 antibody with IgG of the same species). Positive staining appears red, streptavidine-alkaline phosphatase.

Antibody specificity was further verified by Western blotting of proteins extracted from peripheral blood mononuclear cells (MNC), followed by staining of the blot by the primary polyclonal antibody used for immunohistochemistry.¹¹ Briefly MNC were isolated using the standard Ficoll-Hypaque density centrifugation (Pharmacia, Sweden), washed three times in Hanks's balanced salt solution, resuspended in RPMI-1640 Gibco (NY, USA) with 1% fetal calf serum and stimulated with hrIL1 α . After incubation for six hours, at 37°C in an atmosphere of 5% carbon dioxide, the cells were homogenised by repeated freezing. Samples were analysed for MCP-1 by electrophoretic separation on 12% sodium dodecyl sulphate-polyacrylamide gels using the bio-Ra-anti-MCP-1 for detection.

Evaluation of the staining was done in collaboration with a pathologist. The number of positive cells in each area of the vessel wall of each section was assessed by counting up to 200 cells in three adjacent visual fields at a magnification of 250. The areas examined on each section were the intima, media, and adventitia of the vessel wall. All data are presented as positively stained cells as a percentage of all cells counted in each area, expressed as mean and range.

MCP-1 QUANTIFICATION IN PLASMA AND CD4/CD8 RATIO IN PERIPHERAL BLOOD

Heparinised plasma, from which MNC were separated, was frozen and stored at -20°C for later analysis. The method for analysis of MCP-1 was based on a sandwich ELISA kit (RD systems, UK, Cat. No DCP00). Intra-assay and interassay variation, including day to day variation and recovery of the MCP-1 ELISA has been described (Ellingsen T, *et al*, unpublished data).

T lymphocyte subset was determined as already published.¹² Briefly purified MNC suspensions were obtained from heparinised whole blood samples by standard Ficoll-Hypaque density centrifugation. Monocytes were depleted by incubation on glass Petri dishes at 37°C for 45 minutes. T lymphocytes were detected with a monoclonal mouse antibody, OKT3, which identifies all T lymphocytes (CD3+). OKT4 is specific for the T helper subset (CD4+) and OKT8 for the suppressor/cytotoxic subset (CD8+). MNC were incubated with diluted monoclonal antibody at 4°C for 30 minutes, washed three times, pelleted, and incubated with 1:20 of fluoresceinated goat antimouse IgG (Dako, Denmark) for 30 minutes. The cells were washed twice and resuspended, set on slides, and examined by a Leitz microscope with epifluorescence. Percentages of fluorescent positive cells were evaluated by counting a minimum of 200 cells. Monocytes were excluded by morphology in phase contrast.

STATISTICAL ANALYSIS

Differences between groups were analysed by the Mann-Whitney rank test. Data are expressed as mean, median, and range. For correlation analysis the Pearson product

Figure 3 Staining of smooth muscle cells in the media, marked by arrows. Magnification $\times 200$. Positive staining appears red (streptavidine-alkaline phosphatase).

moment test was used. p Values <0.05 were considered significant. All calculations were performed using Sigma Stat.

Results

IMMUNOHISTOCHEMICAL LOCALISATION OF MCP-1 IN THE WALL OF THE TEMPORAL ARTERY

In biopsy specimens of the temporal artery from 12 patients with TA, the localisation of MCP-1 differed among mononuclear cells, smooth muscle cells, endothelial cells, and multinucleated giant cells (fig 1). Table 1 shows the percentages of positively stained cells in the intima, media, and adventitia. Endothelium of both the arteries and vasa vasorum were examined and all biopsy specimens stained positively in the patients with TA (fig 2). In the intima 73% (range 57–91%) of the cells stained positively. In the media 49% (range 32–67%) of the cells judged to be predominantly smooth muscle cells stained positively (fig 3). In the adventitia 74% (range 62–91%) of the cells, predominantly mononuclear cells and fibroblasts, stained positively. Table 1 lists the results of the staining of five patients not fulfilling the criteria of TA and PMR.

The number of positive stained cells was mainly observed around the lamina elastica interna, especially if destruction was present (fig 2a, point B).

Antibody specificity of the biotinylated polyclonal rabbit antibody is illustrated by Western blotting. The polyclonal antibody used for immunohistochemistry has been shown to detect only one band in lane 3, being proteins extracted from IL1 α stimulated MNC. This band equals the staining of hrMCP-1 in lane 2 (fig 4).

PLASMA MCP-1 QUANTIFICATION AND CD4/CD8 RATIO IN PERIPHERAL BLOOD

In the 33 patients with TA, after the temporal arterial biopsy sample was taken and before corticosteroid treatment was started, the level of MCP-1 in plasma was in the range 82–778 pg/ml, corresponding to a mean of 391 pg/ml (table 2).

Figure 4 Bio-Ra-anti-MCP-1 is specific on Western blotting (sodium dodecyl sulphate-polyacrylamide gel electrophoresis using 12% gels) analysing IL1 α stimulated MNC (lane 3), giving only one band equalling hrMCP-1 (lane 2). Lane 1 = molecular weight marker.

Figure 5 Raised levels of plasma MCP-1 were found in untreated patients with temporal arteritis and polymyalgia rheumatica compared with healthy controls.

In the 27 patients with PMR, after temporal arterial biopsy and before corticosteroid treatment was started, the level of MCP-1 in plasma was detected in the range 29–1153 pg/ml, corresponding to a mean of 402 pg/ml (table 2).

In 12 healthy controls, plasma MCP-1 was detected in the range 101–234 pg/ml, corresponding to a mean of 158 pg/ml. Figure 5 illustrates all the ELISA results.

The levels of MCP-1 in plasma from healthy controls were significantly lower than for TA and PMR ($p < 0.001$ in both cases). No significant difference was found between TA and PMR plasma levels of MCP-1 ($p = 0.8$). In both TA and PMR no significant correlation was found between plasma MCP-1 levels and ESR (TA $p = 0.97$, $r = 0.008$; PMR $p = 0.77$, $r = 0.06$), CD4/CD8 ratio (TA $p = 0.63$, $r = -0.10$; PMR $p = 0.89$, $r = 0.03$), or haemoglobin concentration (TA $p = 0.42$, $r = 0.11$; PMR $p = 0.19$, $r = 0.29$).

Discussion

The cause and pathogenesis of TA and PMR still remain unknown.

TA is a focal granulomatous giant cell vasculitis with skip lesions affecting medium and large size arteries, especially branches from the aortic arch, extracranially as well as intracranially. Vascular disease in PMR has recently been described.⁸

Several authors have claimed that PMR is a variant of TA without evidence of arteritic lesions.^{2,13} Lack of vasculitic disease, seen as absence of destruction of the internal lamina elastica membrane and lack of infiltration by mononuclear cells in the arterial wall biopsy specimen, may be due to the nature of the disease being granulomatous with skip lesions.

For leucocyte migration into the vessel wall, local chemotactic factors have to be present to direct the migration.

In patients with TA we found MCP-1 positive cells in the intima, media, and adventitia. Endothelium, some giant cells, some cells with features like smooth muscle fibres, and a large number of the mononuclear cells present in the arterial wall as well as the vasa vasorum stained positively for MCP-1 (figs 1–3). The staining in the intima and media was less in the five subjects with fever, weight loss, anaemia, or raised ESR of unknown origin.

Endothelial cells have been shown to express mRNA for MCP-1 after in vitro stimulation by IL1 and tumour necrosis factor, but not by IL6.¹⁴ Rollins *et al* made the same observations, including that IFN γ in vitro also stimulates MCP-1 production in endothelial cells.¹⁵ In both studies endothelial cells were isolated from human umbilical veins. As IL1 and IFN γ are produced locally within the vessel wall,⁸ induction of MCP-1 synthesis in endothelial cells is likely to occur in the inflammatory lesion in TA. In TA MCP-1 was located both to the vessel itself and also to the vasa vasorum (fig 2). Involvement of the vasa vasorum has been reported in various vasculitic conditions.¹⁶

Smooth muscle cells have also been shown in vitro after stimulation by IL1 to possess the ability of secreting biologically active MCP-1.¹⁷ Thrombin, which is a serine protease released at the sites of injury in vessels, stimulates in vitro expression of transcripts for MCP-1 in human smooth muscle cells in a time and concentration dependent manner.¹⁸ Smooth muscle cells obtained from human atherosclerotic lesions cultured in vitro express the CC-chemokine receptor 2, which has high affinity for the binding for MCP-1.¹⁹

This observation indicates that the smooth muscle cells can participate in inflammatory conditions. It has been claimed that initial events in the inflammatory process in TA involve swelling and degeneration of some smooth muscle cells, as observed by electron microscopy.²⁰ These observations are in agreement with the observation that smooth muscle cells stained positive for MCP-1, indicating participation in the inflammatory process (figs 2 and 3).

The localisation of MCP-1 in endothelial cells in the intimal lesions of atherosclerosis has been described.²¹ Furthermore, MCP-1 could

be detected in macrophages and smooth muscle cells adjacent to the atherosclerotic lesion.²² MCP-1 mRNA were detected in 16% of cells in this area, whereas in normal unaffected vessels transcripts could only be detected in <0.1%.²²

In plasma of both patients with TA and patients with PMR we found significantly raised levels of MCP-1 compared with healthy controls (fig 5). The level of plasma MCP-1 in healthy controls has been found to be similar by other investigators.²³ In 405 healthy subjects aged 20–72 plasma MCP-1 levels were found equalling our measurements—in subjects aged 60–69 the mean (SD) MCP-1 level was 142 (123) pg/ml and in subjects older than 70 the MCP-1 level was 157 (59) pg/ml.²⁴

No significant difference was observed in the levels of MCP-1 measured in plasma for these two conditions. We found no correlation between plasma MCP-1 and the acute phase response in patients with TA and patients with PMR.

Global assessment by the patient or doctor in TA or PMR is not routinely done, though it was proposed by Ellis and Ralston in 1983.²⁵ They used a numeric graded clinical score for assessing the severity of disease evaluated by the doctor. In TA and PMR no single clinical variable is currently used for monitoring clinically assessed disease activity as exemplified by the core set criteria for evaluating disease activity in rheumatoid arthritis.

Others have found a correlation between plasma MCP-1 levels and the clinical course in patients with sarcoidosis.²⁶ This indicates that plasma MCP-1 may be used for monitoring disease activity in conditions with granuloma formation.

In conclusion, our results indicate that MCP-1 participates in the inflammatory process in TA and PMR. Plasma MCP-1 cannot distinguish between TA and PMR. Whether or not plasma MCP-1 levels measured over time will provide information about long term outcome and effect of treatment in TA and PMR is still unknown.

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