Post-onset inhibition of murine arthritis using combined chemokine antagonist therapy

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Objective. To investigate the effect of targeting the chemotaxis of monocytes and polymorphonuclear monocytes (PMNs) in situ in MRL-Fas/lpr arthritis.

Methods. MRL-Fas/lpr mice were injected intradermally with complete Freund’s adjuvant and cellular infiltration into the joint was monitored. Once clinical disease developed, the animals received one of three treatments: MCP-1(9–76); MCP-1(9–76) plus Gro-α(8–73); or control peptide, MCP-1 Ala. The bimalleolar ankle width was measured for 11 days and histological examination of the joints was then assessed.

Results. Cellular infiltration started after the onset of ankle swelling, and increased progressively. The incidence of swelling and the histopathology was reduced after day 6 of treatment in the MCP-1(9–76)-treated mice. Mice treated with the two antagonists MCP-1(9–76) and Gro-α(8–73) displayed a further significant reduction in disease parameters.

Conclusion. Treatment after disease onset with chemotactic antagonists for monocytes and PMNs significantly alleviated both the swelling and the histopathology seen in arthritis, suggesting that chemokine antagonists are an effective anti-inflammatory therapy.

Key words: Arthritis, Chemokines, Antagonists.

Chemokines are chemotaxis-inducing proteins of 70–130 amino acids that are produced by leucocytes and other tissue cells, and they control leucocyte migration during haemopoiesis, innate and adaptive immune responses, and inflammation [1–5]. These small proteins act on target leucocytes expressing complementary chemokine receptors in an autocrine or paracrine manner [6]. Chemokine expression has been found in the joints of rheumatoid arthritis patients, inferring a causal relationship between their production and the inflammatory infiltrate [7, 8], and thus could be promising targets for new therapeutic modalities.

In order to study cellular involvement in the pathology of arthritis, we have developed a mouse model for inflammatory arthritis: complete Freund’s adjuvant (CFA) enhancement of the spontaneous arthritis in MRL-Fas/lpr mice [9]. This model has been used for testing a number of therapeutic modalities [10, 11]. We have reported previously that the MCP-1 antagonist MCP-1(9–76), which binds the CCR2 receptor on monocytes, prevents the development of arthritis if administered at the same time as CFA [11]. We concluded that targeting only monocytes would have limited efficacy in controlling the migration of other leucocyte populations involved in inflammatory disease.

We investigated whether targeting the infiltration of both monocyte and PMN populations had a more pronounced therapeutic effect on established mouse arthritis than targeting monocytes alone.

Materials and methods

Animals

MRL-Fas/lpr mice were obtained from a breeding colony located at the University of British Columbia. This colony was established from stocks originally purchased from the Jackson Laboratories (Bar Harbor, ME, USA) and bred for 2–9 generations. The mice were maintained on a standard diet with water ad libitum. The colony was tested routinely for Mycoplasma pulmonis and M. arthritidis, rodent coronaviruses (including hepatitis viruses) and Sendai virus using the Murine ImmunoComb test (Charles River Laboratories, Wilmington, MA, USA). Only antibodies against coronaviruses were detected. Male and female mice 13–15 weeks of age were pooled from different litters on the basis of sex.

Peptides

MCP-1(9–76), Gro-α(8–73) and MCP-1 Ala, an analogue of MCP-1(9–76) in which all the cysteines are replaced by alanines, were all synthesized chemically and purified as described [12, 13]. The concentration of the antagonists used for therapy was determined as follows. The MCP-1 antagonist competed for binding to MCP-1 receptors with a Kd of 8.3 nM and in vitro inhibited 10 nM of MCP-1 with an ED50 of 70 nM. As the concentration of antagonist required for inhibition of MCP-1 in vivo is unknown, we used a 30-fold excess over the Kd calculated on the basis of an average exchangeable fluid volume of 2 ml per mouse. The concentration of Gro-α(8–73) was determined similarly.

Arthritis induction and treatment

Male and female MRL-Fas/lpr mice (n = 126), 13–15 weeks old, were injected intradermally at two thoracic sites with 0.05 ml CFA supplemented to 10 mg/ml with heat-inactivated, lyophilized Mycobacterium tuberculosis (RA37; Difco Laboratories, Detroit, MI, USA) as described previously [9]. Once the animals had clinical disease, as determined by significant footpad swelling, they were selected randomly from the bulk storage cages and divided into three treatment groups, each of 42 animals. The bimalleolar ankle width was measured on day 1 and every 2 days thereafter. Once clinical disease developed, the animals received one of three treatments: MCP-1(9–76); MCP-1(9–76) plus Gro-α(8–73); or control peptide, MCP-1 Ala. The bimalleolar ankle width was measured for 11 days and histological examination of the joints was then assessed.

Results. Cellular infiltration started after the onset of ankle swelling, and increased progressively. The incidence of swelling and the histopathology was reduced after day 6 of treatment in the MCP-1(9–76)-treated mice. Mice treated with the two antagonists MCP-1(9–76) and Gro-α(8–73) displayed a further significant reduction in disease parameters.

Conclusion. Treatment after disease onset with chemotactic antagonists for monocytes and PMNs significantly alleviated both the swelling and the histopathology seen in arthritis, suggesting that chemokine antagonists are an effective anti-inflammatory therapy.
into the four experimental groups (keeping equal numbers of males and females per group) and treated as follows. In group 1 (n = 54), six mice were killed at the time of swelling (t = 0) and on days 1, 2, 4, 6, 7 after swelling, and the hind paws were removed into buffered formalin for histological and immunohistological staining. Group 2 mice (n = 9) were treated with MCP-1(9–76), 2 mg/kg i.p. daily for 12 days. Group 3 mice (n = 8) were treated with MCP-1 + Gro-α(8–73), 2 mg/kg of each antagonist i.p. daily for 12 days. Group 4 mice (n = 14) were treated with the control antagonist MCP-1 Ala 2 mg/kg, injected i.p. daily for 12 days. Bimalleolar ankle width was measured in all animals every day for 12 days using a micrometer. At the end of 12 days the hind paws were removed and placed in buffered formalin for histological staining.

Clinical and histological examination of joints

The presence of clinical disease (visual appearance of arthritis) was evaluated every day and scored as positive if erythema and swelling of a fore or hind paw was observed. Approximately 70% of animals developed swelling 13–21 days after CFA injection, allowing their inclusion in the study.

For histological evaluation of the arthritis, the skin was removed from the joints and the joints were placed in 10% formic acid for 48 h for decalcification, and processed for paraffin embedding. Serial sections were cut to a thickness of 5 μm and stained with haematoxylin and eosin. A blinded observer examined sections. The following parameters were graded 0–2: (i) subsynovial inflammation (0, normal; 1, focal inflammatory infiltrates; 2, inflammatory infiltrate dominated the cellular histology); (ii) synovial hyperplasia (0, normal; 1, a continuous synovial lining of one joint at least three layers thick; 2, synovial lining at least three layers thick detected in several joints); (iii) cartilage erosion and pannus formation (0, normal; 1, pannus partially covered the cartilage surfaces without evident cartilage loss; 2, pannus connected to evident cartilage loss); (iv) bone destruction (0, normal; 1, detectable destruction of bone by pannus or osteoclast activity; 2, pannus or osteoclast activity had destroyed a significant part of the bone) [9]. Histopathological changes were scored for the left and right legs. The final score for each parameter was the average of the score for the mouse’s left and right legs. The total score for each mouse represented the overall inflammation score and was the total of the above three parameters.

For immunohistological evaluation of the cellular infiltration, the skin was removed from the joints and the joints were placed in zinc fixative for 8–12 h and processed for paraffin embedding. Serial sections were cut to a thickness of 5 μm. Paraffin was removed from the sections, endogenous peroxidase was blocked, and the sections were stained with MCA 771G and F4/80 (Serotec, Raleigh, NC, USA) according to the manufacturer’s instructions. Scores were assigned as follows: for subsynovial infiltration, 1 = normal, no cellular infiltration, 2 = a few cells infiltrated, 3 = many cells infiltrated; for connective tissue infiltration, 1 = normal, no cellular infiltration, 2 = a few cells infiltrated, 3 = many cells infiltrated. As above, the left and right legs were both scored, and the final infiltration score for each mouse represented the sum of the scores for the subsynovium and connective tissue.

Statistical analyses

Paired sets of ankle width measurements and histopathological indices in the three groups were compared by analysis of variance and Bonferroni multiple comparison tests.

Results

Kinetics of cell infiltration vs arthritis symptoms

Figure 1A demonstrates that histopathological scores of the synovial lining, subsynovium and connective tissue increased with time after the onset of swelling. Immunohistochemical staining indicated a significant neutrophil presence in the connective tissue 4 days after the onset of swelling. However, monocytes/macrophages migrated into the subsynovium 7 days after the onset of swelling (Fig. 1B).

Chemokine antagonists inhibit arthritis after onset of swelling

It can be seen from Fig. 2A and B that swelling was temporally reduced 2 days after treatment in all groups. However, treatment with CCR2 antagonist delayed relapse, reducing both swelling and histopathological scores. When both antagonists were applied together, the inhibition of these disease parameters was significantly greater than when the antagonists were applied alone.

Discussion

Chemokines are associated with the manifestation of rheumatic diseases. Biopsies of rheumatoid arthritis patients showed that chemokines such as MCP-1, interleukin8 and RANTES [regulated upon activation, normal T-cell expressed and secreted chemokine] are expressed in the inflamed tissue [7, 8].
Furthermore, recent work from our laboratory has shown that systemic treatment of MRL-Faslpr mice with MCP-1, RANTES or MIP-2 can increase severity of spontaneous arthritis in mice, suggesting that these chemokines play a critical role in the disease process (J.-H. Gong, J.D. Waterfield and I. Clark-Lewis, unpublished observations).

Our laboratory has developed antagonists for MCP-1 and Gro-α and characterized their inhibitory properties in cell lines and freshly isolated leucocyte subpopulations. A number of studies, including ours, show that chemokine antagonists can prevent disease onset [11, 13]. However, it is more important to determine whether chemokine antagonists will be useful as a therapeutic modality for treatment of existing disease.

Cell infiltration is believed to be an early event of inflammation. However, in this study the early symptom, tissue swelling, appears first, followed by cellular infiltration. Neutrophils moved in much sooner (4 days after swelling) than monocytes (7 days after swelling). The cellular infiltration increased gradually and reached a maximum after 6–7 days. After the onset of swelling, treatment with CCR2 antagonists (monocytes) reduced disease severity while CCR2 (monocytes) and CXCR2 (PMNs) antagonists administered together had a significantly more pronounced effect. It was interesting to note that the MCP-1 Ala control peptide (a biologically inactive form of the antagonist [12]) had an anti-inflammatory effect for up to 5 days after injection. As this response has been noted in untreated animals, we do not feel that this is a direct consequence of the control antagonist but rather the transient effect of endocrine involvement on the immune system, initiated by a stress response to therapy.

A confounding problem that needs to be solved when considering the targeting of chemokines is the apparent redundancy of the chemokine family [14]. It is well known that different chemokines are produced by a number of cells in inflamed tissue. Moreover, each target cell expresses several types of chemokine receptors. In the past it was unclear whether therapy targeting a single chemokine/receptor would be doomed to failure, as other chemokines with related biological effects could compensate for its inactivation [6, 14–16]. Gene knockout studies show that deletion of individual chemokines/receptors in situ effectively suppresses inflammation [17]. Our findings also address the issue of the redundancy of chemokines in situ. We have clearly blocked cellular infiltration using one chemokine antagonist for each subpopulation. A possible explanation for this finding is that chemokines may be functioning in a multistep navigational manner, whereby disruption of one component of the pathway will unhinge further leucocyte migration. This would occur when there was not enough redundant chemokine produced to override the effect of the applied antagonist. It will require further experimentation to determine whether such lack of redundancy is due to regional differences in the synthesis of chemokines. Regardless, redundancy should still be considered an important issue when designing drugs targeting chemokines and their receptors.

Our findings clearly demonstrate that drugs that antagonize chemokines can reduce inflammation even when administered after the onset of disease symptoms. Thus, in the clinical setting, chemokine antagonists can be useful not only for preventing but also for the treatment of arthritis, provided the drugs are applied at an early stage of inflammation.

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Conflict of interest

The authors have declared no conflicts of interest.
References