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MONOCYTE CHEMOTACTIC PROTEIN-1 INHIBITS THE INDUCTION OF NITRIC OXIDE SYNTHASE IN J774 CELLS

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We evaluated the effect of monocyte chemotactic protein-1 (MCP-1) on the induction of nitric oxide synthase activity in J774 cells. MCP-1 was able to inhibit the production of nitric oxide induced by LPS and IFN-γ in a dose-dependent manner. Moreover, the inhibition was only achieved when the cells were pretrated with MCP-1. No inhibition was observed when MCP-1 was added after stimulation with LPS and IFN-γ. These results demostrate that MCP-1 is able to inhibit the induction of nitric oxide synthesis.

Nitric oxide (NO) is synthetized from L-arginine by nitric oxide synthase. At present two isoforms have been isolated and characterized; the first one is Ca^{+2}- and calmodulin-dependent being responsible for the constitutive low-output pathway. NO-derived from this pathway functions as an inter- and intra-cellular signal (1,2). The second one is Ca^{+2}-independent and it is inducible by citokines and bacterial products and accounts for the high-output pathway (3,4).

During the activation of murine macrophages, the induction of the high-output nitric oxide pathway has been pointed out as a very important mechanism for controlling proliferation of neoplastic cells (5,6) as well as certain microbial pathogens, particulary intracellular microorganisms (7,8).

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However, cytokines can also down-regulate NO synthesis as demostrated for IL-4 and IL-10 (9,10). The same activity has been reported for the three members of the trasforming growth factor β-family (TGF-β1, -β2 and -β3) (11) as well as for epidermal growth factor (12).

In this context IL-8, a neutrophil chemoattractant cytokine is also able to inhibit the induction of the nitric oxide synthase (13).

MCP-1 is a potent chemoattractant for monocytes and belongs to a novel family of proinflammatory proteins also known as chemokines (14). Chemokines are devided into two different branches (α and β families) by the position of the first two closely paired cysteines. The first one, to it belongs IL-8, has an intervening amino acid between two cysteines (C-X-C), whereas members of the β-family (MCP-1) have a C-C configuration.

In the present study we investigated whether the monocyte chemotactic protein-1 (MCP-1) has any effect on the production of nitric oxide by an activated murine macrophage cell line.

Materials and Methods

Materials: Murine recombinant IFN-γ and N^G-monomethyl-L-arginine (L-NMMA) were kindly suplied by Dr. S. Moncada (Wellcome Research Labs, UK). Lipolysaccharide (*E.coli* 0111:B4) was obtained from Sigma. Human recombinant monocyte chemotactic protein-1 (MCP-1) was a gift from Dr. A. Mantovani (Istituto di Ricerche Farmacologiche Mario Negri, Italy).

Cell culture: J 774 monocytic leukemia cells were grown in RPMI 1640 medium containing 10 % foetal calf serum, 100 U/ml penicillin, 100 ug/ml streptomycin and supplemented with L-glutamine (2mM). Cells were cultured at 37°C in an humidified atmosphere of 5 % CO2, in 24-well plates at 10^6 cells/ml. MCP-1, at different concentrations, was added at -18, -6, 0 or + 6 hours relative to the addition of LPS (10 ng/ml plus IFN-γ (50U/ml). Culture supernatants were monitored for NO_2^- levels after incubation for 24 hours.

Nitrite assay: Nitrite level in cell-free culture supernatants were determined using the spectrophotometric method based on the Griess reaction, as described (15).

Results

As expected, J774 cells released significant amounts of NO, measured as NO_2^-, when stimulated with LPS and

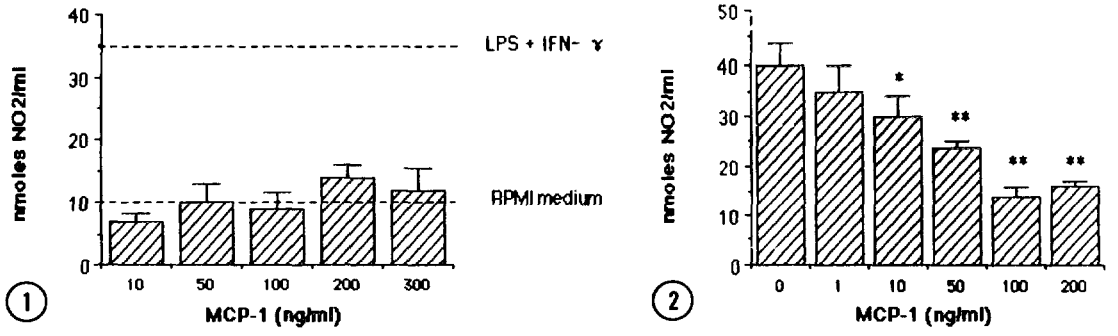

Figure 1.
Effect of MCP-1 on nitric oxide synthesis. MCP-1 at different concentration was added to J774 cells and nitrite levels were determined after 30 hours of incubation. Data are means± SEM of three experiments.

Figure 2.
Dose-dependent inhibition of LPS and IFN-γ induced nitric oxide synthesis by MCP-1.
LPS (10 ng/ml) and IFN-γ (50U/ml). Data are means ± SEM of three experiments. * $p < 0.05$, ** $p < 0.01$ by Student's t test.

IFN-γ. Such a NO_2^- production is practically abrogated when the NO-pathway inhibitor, L-NMMA, is added to the medium.

No induction of NO synthesis was achieved when MCP-1 alone was added to cell cultures at 10, 50, 100, 200 and 300 ng/ml for up to 30 hrs (see figure 1).

As shown in figure 2, MCP-1 was able to inhibit (50 % inhibitory concentration, IC_{50} of 30 ng/ml) in a dose-dependent manner the NO_2^- released induced by the treatment of LPS and IFN-γ when cells were pretreated for 18 hours with MCP-1. Inhibition was only achieved when concentration was higher than 10 ng/ml. Boiled MCP-1 did not has any inhibitory effect on NO synthesis (data not shown).

In addition, the inhibition of NO synthesis was also time-dependent (see figure 3) being maximal when MCP-1 was added 18 hours before LPS and IFN-γ and in a lesser extention, when added at the same time of stimuli. It should be noted that the addition of MCP-1 6 hours after the addition of stimuli did not produce any decrease in NO_2^- release.

As previously reported, the time-course of NO synthesis, measured as NO_2^- , indicates that synthesis starts after a lag phase of 6-8 hours after stimulation, just the

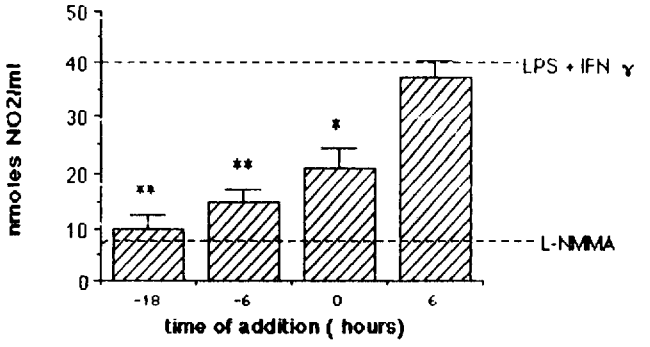

Figure 3.
Time-dependent inhibition of nitric oxide synthesis by MCP-1.
MCP-1 (100 ng/ml) was added to J774 cells at different times relative to the addition (time 0) of LPS (10 ng/ml)and IFN-γ (50 U/ml). Nitrite levels were evaluated after 24 hours of incubation. L-NMMA was used at the concentration of 200 μM. Data are means ± SEM of three experiments. * $p < 0.05$, ** $p < 0.01$ by Student's t test.

time needed for the novo protein synthesis (16). Therefore, MCP-1 seems to have weak effects once the induction is started because when added at the same time of stimuli, inhibition was achieved but much less effective.

Discussion

MCP-1 is a potent chemoattractant for monocytes and belongs to a novel family of proinflammatory proteins also known as chemokines (14). Several lines of evidences suggest that MCP-1 is an important determinant of macrophages infiltration into tumors (17,18).
Although the biological significance of these findings is not clear, it should be mentioned that several tumors have been shown to release MCP-1, therefore it might play a role in tumor biology (19).
On the other hand, tumor-associated macrophages represent a very complex association in which macrophages seem to contribute with tumor growth factors and to other important processess for tumor biology such as fibrin deposition (20, 21). However macrophages, when activated, are able to inhibit tumor growth by several mechanisms. Among them, NO seems to be a very important cytotoxic effector molecule (5,6). Very recently, it has

been proposed that the intratumor macrophage arginine metabolism (through the NO and arginase pathways) is a molecular explanation for the dual ability of the immune system to inhibit or stimulate tumor growth, in dependence of whether NO/citrulline or ornithine/urea formation is taken place (22). At this point, tumor-derived MCP-1 is likely to represent a mechanism for controlling NO-mediated macrophage cytotoxicity. It has been previously reported for tumor-associated macrophages a decreased tumoricidal activity, in the absence of further stimulation (23, 24, 25). However, it is obvious that the maintenance of this association as a symbiotic state should be under a very restricted control, and the identification of activating signals might have important pharmacological implications.

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