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Effect of mast cell granules on the gene expression of nitric oxide synthase and tumour necrosis factor-α in macrophages

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PREVIOUS studies have shown that mast cell granules (MCG) inhibit numerous macrophage functions including tumour cytotoxicity, superoxide and nitric oxide (NO) production, and FCγ2a receptor-mediated phagocytosis. In this study, the effect of MCG on macrophage TNFα and nitric oxide synthase (iNOS) mRNA expression, and the production and fate of TNFα were examined. Upon activation with LPS+IFNγ, macrophages expressed both TNFα and iNOS mRNA and produced both TNFα and NO. Co-incubation of LPS+IFNγ-activated macrophages with MCG resulted in dose-dependent inhibition of iNOS mRNA expression. TNFα production in the activated macrophages was decreased by MCG, which was associated with a reduction in TNFα mRNA expression. MCG were also capable of degrading both macrophage-generated and recombinant TNFα. The direct effect of MCG on TNFα was partially reversed by a mixture of protease inhibitors. These results demonstrate that MCG decrease the production of NO and TNFα by inhibiting macrophage iNOS and TNFα gene expression. Furthermore, MCG post-transcriptionally alter TNFα levels via proteolytic degradation.

Key words: Nitric oxide, TNFα, Macrophages, Mast cell granules, Proteases

Introduction

Macrophages play a major role in microbicidal and tumoricidal activity, antigen presentation, and the production of a variety of cytokines and inflammatory mediators.[1,2] Mast cells, on the other hand, play a key role in hypersensitivity reactions by secreting mediators such as histamine, proteoglycans, various cytokines, metabolites of arachidonate and unique proteases.[3–6] The recruitment of macrophages to sites of mast cell degranulation and the subsequent phagocytosis of granules *in vivo* was first reported by Fawcett.[7] We have previously shown that mast cell granules (MCG) interact with rodent macrophages and downregulate superoxide production,[8–10] tumour cell killing and NO production.[11] These reports confirm that mast cell-macrophage communication has a regulatory effect on host defence mechanisms and inflammation. A role for mast cells in tumour growth and metastasis is evident by the presence of an increased number of mast cells at the periphery of certain tumours[12–14] and by the angiogenic activity of several mast cell products.[15,16] Similarly, macrophage products such as NO and TNFα are implicated in tumour growth and inflammation. The fact that increased number of mast cells are found in the periphery of tumours and MCG interaction inhibited macrophage-mediated tumour cell killing prompted us to examine the mechanism of the effect of MCG on macrophage NO and TNFα production. The focus of this study was, therefore, to examine the effect of MCG on mRNA expression of iNOS and TNFα in macrophages. The results demonstrate that MCG interact with macrophages, inhibit iNOS and TNFα mRNA expression, and degrade TNFα.

Materials and methods

Materials

Lipopolysaccharide (LPS) from *Escherichia coli* cell wall, penicillin, streptomycin, HEPES, metrizamide, leupeptin, pepstatin A, aprotinin, phenylmethylsulfonyl fluoride (PMSF) and thiazolyl blue dye (MTT) were purchased from Sigma Chemical Co. (St Louis, MO). Soybean trypsin inhibitor was purchased from Boehringer Mannheim (Indianapolis, IN). Heparin solution was supplied by Elkins-Sinn, Inc. (Cherry Hill, NJ). Minimum essential medium with Earle's salts (MEM) and fetal bovine serum were obtained from Hyclone Laboratories (Logan, UT). The ELISA kit for mouse TNFα was purchased from Genzyme (Cambridge, MA). Plastek M cell culture plates were from Mat Tek Co. (Ashland, MA). The RNA Stat-60 kit was purchased from Tel-Test, Inc. (Friendswood, TX). All tissue culture reagents used in this study were free from detectable levels of endotoxin (<25 pg/ml) when tested by the limulus amoebocyte lysate gel-clot assay (Associates of Cape Cod, Inc., Woods Hole, MA).

Animals

Male Sprague-Dawley rats (350–400 g) used for harvesting mast cells, and male C57BL/6J mice (2–3 months old), the source of macrophages, were purchased from Harlan Co., Indianapolis, Indiana.

Harvesting and culture of murine peritoneal macrophages

Three days prior to harvesting peritoneal exudates, each mouse was injected intraperitoneally with 1.5 ml of a sterile solution of proteose peptone (10% w/v). The peritoneal cavity of each mouse was lavaged twice with 2.5 ml of minimum essential medium containing 15 mM HEPES, 100 units ml of penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum (HMEM) and 5 units/ml of heparin. The pooled cell suspension was sedimented by centrifugation at 250 × ***g*** for 10 min, washed twice and resuspended in HMEM without heparin. Aliquots of the cell suspension containing 2×10^5 cells were seeded in each well of a 96-well culture plate. Non-adherent cells were removed by washing after 2–4 h of incubation at 37°C. Macrophages were activated with LPS (100 ng/ml) + IFNγ (10 units/ml) in all experiments, since this concentrations have been found to induce optimum activation.

Isolation of mast cells

The method employed for the isolation of mast cells has been previously described.[11,17] In brief, mast cells were collected by lavage of the peritoneal and thoracic cavities of adult rats with 50 ml HMEM containing 5 units/ml heparin. The lavaged cells from all animals were pooled, centrifuged at 250 × ***g*** for 10 min at room temperature, and washed twice with HMEM. Two ml of the cell suspension containing 6–8 $\times 10^7$ cells were gently layered on a 3 ml cushion of 22.5% (w/v) metrizamide (density 1.125 g/ml) in HMEM in a 15 ml centrifuge tube, and centrifuged at 200 × ***g*** for 15 min at room temperature. Mast cells were sedimented at the bottom of the conical tube while other cells (predominantly macrophages) collected at the interface. The mast cell fractions were collected, washed twice, and resuspended in HMEM without heparin. Purity and viability of mast cells isolated by this procedure exceeded 95%.

Preparation of MCG and MCG sonicate

Under sterile conditions at 0–4°C, MCG were prepared from metrizamide-purified mast cells by controlled sonication and sucrose gradient centrifugation. Mast cells were suspended in 1 ml of HMEM and sonicated twice for 20 s with a microtip sonicator (Sonifier Cell Disruptor, model W140) at a power setting of 2.5 and temperature of 4°C.[9,18] The disrupted cells were incubated at 30°C for 15 min and mixed vigorously for 1 min. The sonicate was layered on 2 ml of 0.34 M sucrose and centrifuged at 50 × ***g*** for 10 min at 4°C. The granules at the interface were collected and sedimented by centrifugation at 1800 × ***g*** for 20 min at 4°C. The resulting pellet consisting of a homogeneous preparation of MCG was washed twice and resuspended in the culture medium. The recovery of granules isolated by this procedure ranged from 60% to 80% based on the histamine content of the starting mast cells.

MCG-sonicate was prepared by sonicating purified MCG in HMEM three times for 30 s at maximum power. The quantity of MCG and MCG-sonicate used in each experiment was expressed as the equivalent of the starting mast cell number.

Assay of TNFα

The concentration of TNFα in the culture medium was assayed by both ELISA and bioassay. The sensitivity of ELISA is 31 pg/ml using the protocol recommended by the manufacturer. For the bioassay, the target L929 cells in RPMI-1640 medium were seeded (4.2×10^4 per well) in a 96-well culture plate. After 18 h of culture, medium was replaced with 5 μg/ml of dactinomycin in RPMI. Two to 4 h after incubation with dactinomycin, serially diluted samples and TNFα standards (2–250 pg/ml) were added and incubated for an additional 18–24 h. The medium was then removed, and 1 mg/ml of MTT dye in RPMI (without serum and phenol red) was added. After 2–3 h of incubation, the medium was aspirated completely, and 100 μl of 2-propanol was added to each well. The plate was agitated for 10 min on an orbital shaker and read on a microtitre plate reader at 595 nm.

Analysis of iNOS and TNFα mRNA expression

Mouse peritoneal cells which contained >85% macrophages were seeded in a 10 cm Petri dish and incubated for 4 h to allow for macrophage adherence. After washing three times with HMEM, the cells were treated with MCG and activated with LPS + IFNγ. At selected time points after activation, culture medium was removed, and the total cellular RNA was extracted using an RNA extraction kit. Total RNA (10 μg) was electrophoresed on 0.8% agarose-formaldehyde gel and then transferred to Nytran nylon membrane. After 2 h of prehybridization, the membrane was hybridized with ^{32}P-labelled cDNA probe specific for murine iNOS, TNFα or β-actin. The probes were labelled by random hexamer priming method using [α-^{32}P] dCTP as previously described.[19] The membrane was then washed four times and autoradiographed on a Kodak X-OMAT-AR film. A scanner was used to determine the density of the mRNA band and the relative density of each band was normalized to β-actin.

Treatment of MCG with protease inhibitors

MCG were incubated with a mixture of protease inhibitors containing PMSF 2.5 mM, pepstatin A 5 μg/ml, trypsin inhibitor 50 μg/ml, leupeptin 50 μg/ml, and aprotinin 50 μg/ml, at 37°C for 2 h. The protease inhibitor-treated MCG were then evaluated for their effects on macrophages.

Statistical analysis

Whenever applicable, the data were analysed by one-way analysis of variance with subsequent Student-Newman-Kuels' test. All results were expressed as means ± SEM and $P<0.05$ was considered significant.

Results

Effects of MCG on macrophage iNOS mRNA expression

Earlier work demonstrated that MCG inhibit macrophage NO production.[11] To investigate the mechanism, the effect of MCG on macrophage iNOS mRNA expression was analysed at selected mast cell-to-macrophage ratios. The addition of MCG simultaneously with LPS+IFNγ to macrophage monolayer inhibited iNOS mRNA expression in a dose-dependent manner (Fig. 1). Maximal inhibition was noted at a MCG dose equivalent to a mast cell-to-macrophage ratio of 1:5 and the inhibition was evident even at a ratio of 1:40. When macrophages were pre-incubated with MCG for 3 h prior to activation, the inhibitory effect of MCG decreased compared with simultaneous addition of MCG and the activators. Furthermore, if macrophages were incubated with MCG for more than 6 h prior to activation, there was no inhibition of the expression of iNOS transcript (Fig. 2).

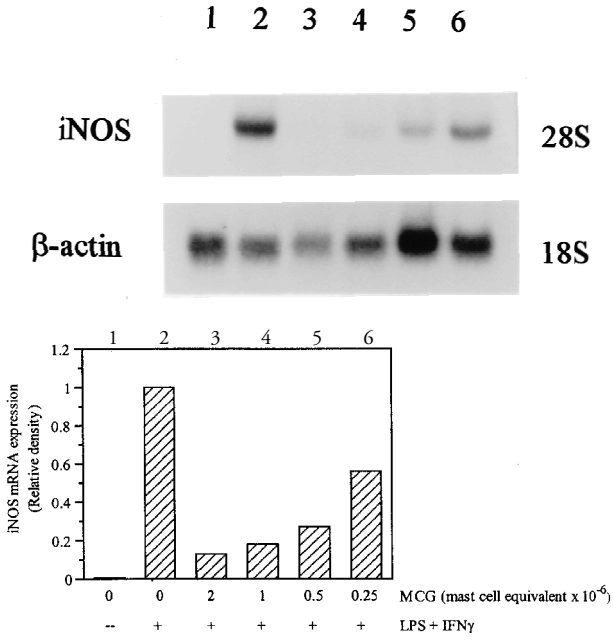

FIG. 1. Effect of MCG on macrophage iNOS mRNA expression. Adherent macrophages (1×10^7) were exposed to the indicated doses of MCG (mast cell equivalent) and activated with LPS (100 ng/ml) + IFNγ (10 units/ml). In all subsequent experiments the macrophages were activated with these concentrations. Eighteen hours after activation, iNOS and β-actin mRNA were analysed by Northern blotting. The β-actin expression was used to normalize the data. For top panel: lane 1, control; lane 2. LPS+IFNγ; lane 3. LPS+IFNγ +MCG 2×10^6; lane 4. LPS+IFNγ +MCG 1×10^6; lane 5. LPS+FNγ +MCG 0.5×10^6; lane 6. LPS+IFNγ +MCG 0.25×10^6. This is representative of three experiments.

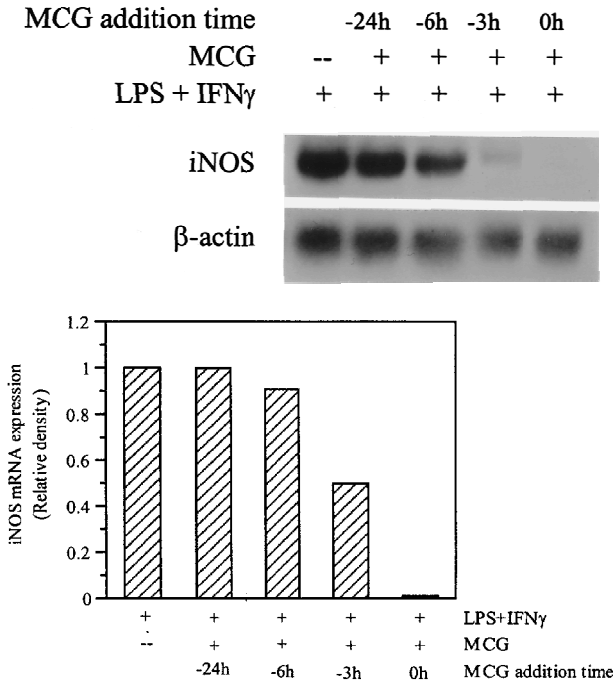

FIG. 2. Effect of pre-treatment of macrophages with MCG on iNOS mRNA expression. Macrophages (1×10^7) were cultured with MCG (2×10^6 mast cell equivalent) for 24, 6 and 3 h prior to activation or added simultaneously with LPS + IFNγ. Eighteen hours after activation, iNOS and β-actin mRNA were analysed by Northern blotting. The β-actin expression was used to normalize the data. This is representative of three experiments.

Effects of MCG on TNFα production

When activated with LPS+IFNγ, macrophages generated large amounts of TNFα. Addition of MCG or MCG-S at the time of LPS+IFNγ stimulation resulted in dose-dependent decrease in TNFα levels and more than a 90% decrease was noted at a MCG dose equivalent to a mast cell-to-macrophage ratio of 1.5 to 2 (Fig. 3). The MCG-induced decrease in TNFα levels was seen when determined by ELISA (Fig. 3A) and by bioassay (Fig. 3B). Although the assayed values differed for the same samples depending on the assay method employed, the decrease of TNFα levels was evident. There was no statistically significant difference between the effects of MCG and MCG-S. Therefore, in later experiments only the intact MCG were used. Unactivated macrophages or those

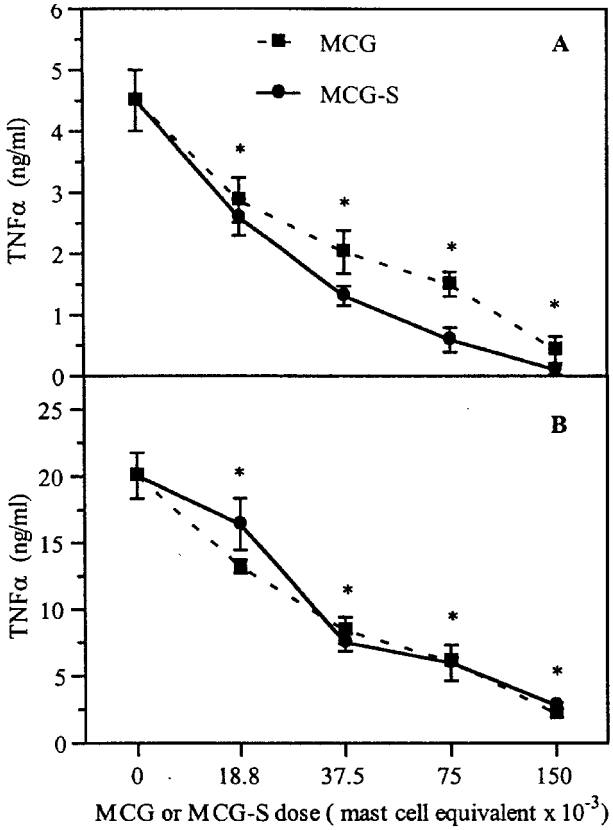

FIG. 3. Dose response effect of MCG or MCG-S on macrophage TNFα production. Adherent macrophages (200,000) were cultured with LPS + IFNγ in the presence or absence of the indicated doses of MCG or MCG-S (mast cell equivalent). After 6h of culture, media were collected and TNFα levels were measured by ELISA (panel A) and bio-assay (panel B) utilizing L929 cells as the target. Each value presented is the mean ± SEM of quadruplicate determinations. There is no significant difference between the effects of MCG and MCG-S. This is representative of three experiments. *P<0.05 vs. no MCG added.

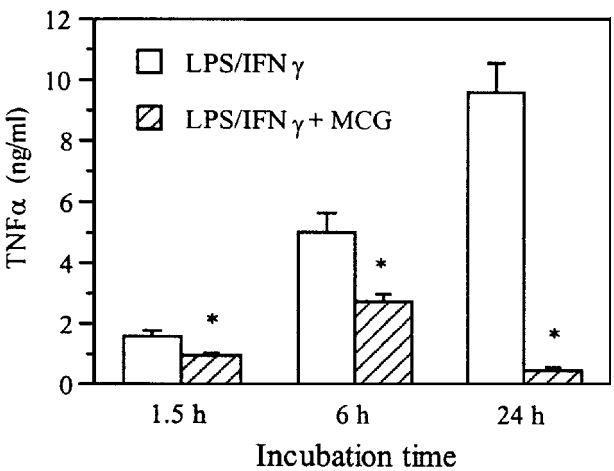

FIG. 4. Time course of the effect of MCG on macrophage secreted TNFα levels. Adherent macrophages (200,000) were cultured with LPS + IFNγ in the presence or absence of MCG (100 000 mast cell equivalent). After the indicated times, media were collected and TNFα levels were measured by ELISA. Each value presented is the mean ± SEM of quadruplicate determinations. This is representative of two experiments. *P<0.05 vs. LPS+IFNγ only at the same time point.

exposed only to MCG or MCG-S did not produce detectable levels of TNFα.

The production of TNFα by macrophages was evident as early as 1.5 h after activation. The cumulative TNFα level increased for up to 24 h while the rate of production appeared to decrease (Fig. 4). The presence of MCG decreased the level of TNFα by approximately 40% at 1.5 and 6 h in contrast to a 95% decrease at 24 h. This effect was also evident when TNFα was assayed by bioassay (data not shown).

Effects of MCG on TNFα mRNA expression

In order to assess the effect of MCG on TNFα mRNA expression by activated macrophages, the transcripts were analysed at 2, 6 and 24 h of incubation after activation. The result demonstrates that TNFα mRNA was rapidly expressed to a maximal level within 2 h after activation and then declined thereafter (Fig. 5). Addition of MCG to LPS+IFNγ-stimulated macrophages inhibited TNFα mRNA expression by 60% at a mast cell-to-macrophage ratio of 1:3.

Effect of MCG proteases on TNFα degradation

Rat mast cell granules contain a variety of proteases including tryptase, chymase, and carboxypepti-

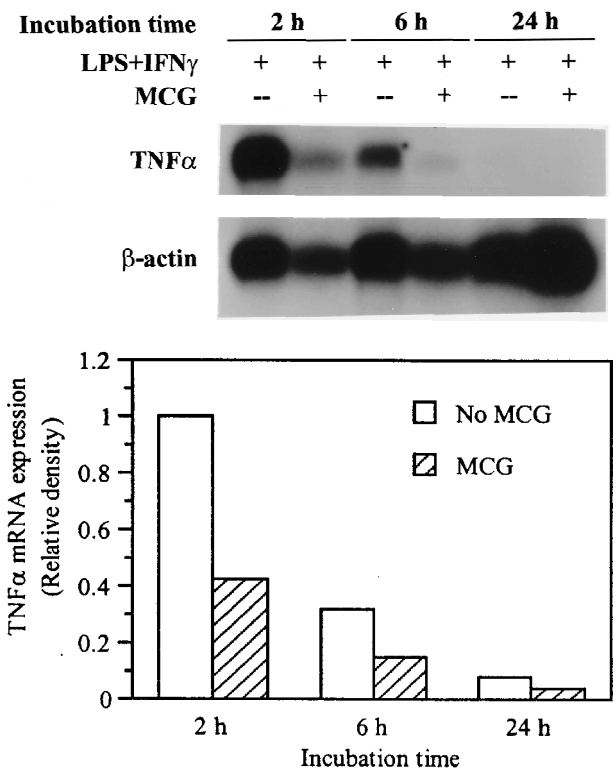

FIG. 5. Effect of MCG on macrophage TNFα mRNA expression. Macrophages (1.5×10^7) were cultured without or with MCG (5×10^6 mast cell equivalent) and simultaneously activated with LPS + IFNγ. After the indicated culture times, TNFα and β-actin mRNA were analysed by Northern blotting. The β-actin expression was used to normalize the data. This is representative of three experiments.

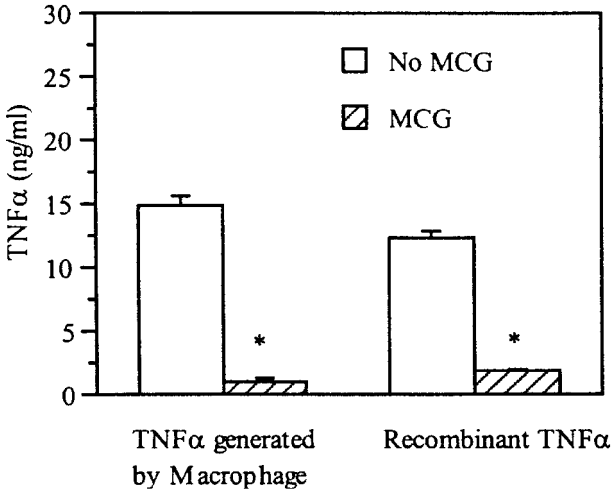

FIG. 6. TNFα degradation by MCG. Macrophage derived and recombinant TNFα were incubated with MCG (100,000 mast cell equivalent) in a volume of 0.2 ml culture medium for 24 h. TNFα was assayed by ELISA. Values presented are the mean ± SEM of quadruplicate determinations. This is a representative of four experiments. * $P<0.05$ vs. no MCG.

dase.[20–23] To investigate if TNFα is susceptible to these enzymes, conditioned media with known levels of TNFα from LPS+IFNγ-activated macrophages or recombinant mouse TNFα were incubated with MCG for 24 h at 37°C. The TNFα levels, assayed by ELISA, indicate that both recombinant and macrophage-secreted TNFα were degraded by MCG (Fig. 6). This effect was partially reversed by the pre-treatment of MCG with a mixture of protease inhibitors containing

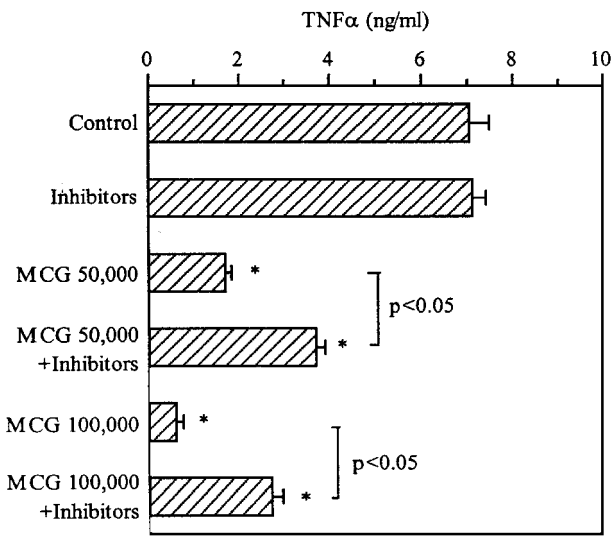

FIG. 7. Effect of protease inhibitors on TNFα degradation by MCG. Mouse recombinant TNFα was incubated with the indicated doses of MCG in cell culture medium for 24 h. MCG were incubated with the protease inhibitor mixture at 37°C for 2 h before being added to TNFα. The TNFα levels were assayed by ELISA. Values presented are the mean ± SEM of quadruplicate determinations. The protease inhibitor mixture contains 2.5 mM PMSF, 5 μg/ml pepstatin A, 50 μg/ml each of trypsin inhibitor, leupeptin and aprotinin. This is representative of three experiments. * $P<0.05$ vs. control.

PMSF, pepstatin A, trypsin inhibitor, leupeptin and aprotinin (Fig. 7). The complete abrogation of TNFα degradation by MCG could not be achieved even after extending the pre-treatment of MCG with these protease inhibitors to 24 h at 4°C.

Discussion

The present study demonstrates that incubation of macrophages with MCG during activation with LPS+INFγ results in the inhibition of iNOS and TNFα mRNA expression with a consequent decrease in NO and TNFα production. This corroborates our previous report documenting MCG inhibition of NO production and tumour cell killing by LPS+INFγ-activated macrophages.[11] The MCG effect was evident even at an estimated mast cell to macrophage ratio of 1:40, a relationship likely to occur *in vivo*. The inhibitory effect of MCG on macrophage iNOS mRNA expression seems transient because this effect disappears when the macrophages were pre-incubated with MCG for 6 h or more. The same phenomenon was observed with the inhibitory effect of MCG on TNFα mRNA expression (data not shown). Our earlier studies have ruled out the possibilities that MCG may inactivate LPS or IFNγ. Further investigation is needed to clarify the mechanism by which MCG inhibit macrophage iNOS and TNFα mRNA expression in a transient manner.

The expression of TNFα by activated macrophages is of particular significance since the genes encoding iNOS and TNFα can be coordinately regulated. *In vivo* studies have suggested a role for TNFα in the positive regulation of iNOS following LPS injection. Anti-TNFα antibodies or soluble TNFα receptor antagonists partially block LPS-induced pulmonary iNOS activity or hepatic iNOS mRNA expression respectively.[24,25] Furthermore, the involvement of TNFα in both the induction and maintenance of iNOS mRNA in macrophages was recently reported using an *in vivo* mouse model.[26] These studies indicate that TNFα is an autocrine regulator of iNOS expression. The TNFα mRNA expression begins as early as 0.5 h,[27] whereas iNOS mRNA cannot be detected until 8 h after activation.[28] Our data also show that TNFα mRNA expression by macrophages is optimal at 2 h after activation and then progressively decreases. Based on those data we conclude that the depletion of macrophage-secreted TNFα by MCG could contribute to inhibition of LPS-induced iNOS mRNA expression. It is clear that the MCG to macrophage ratio that completely inhibits the expression of iNOS mRNA only partially inhibits the expression of TNFα mRNA. We do not yet have an explanation for this phenomenon. It is possible that the iNOS expression needs a finite amount of TNFα to trigger the gene.

Rodent mast cells contain histamine, serotonin, and proteases including serine proteases, neutral

proteases, and carboxypeptidase A.[20–23] Histamine and serotonin at concentrations present in mast cells (20 μg and 2 μg per million cells respectively) may induce immunomodulatory effects. For instance, histamine up-regulates macrophage synthesis of IL-1.[29] However, histamine at concentrations in the range of 10^{-6}–10^{-3} M (equalling and exceeding that present in MCG used in this study) failed to affect TNFα and NO production by macrophages (data not shown). These results are in agreement with our earlier report of MCG inhibition of macrophage superoxide production, which showed that unlike MCG, histamine and serotonin were ineffective in modulating the respiratory burst.[9] The effect of serotonin on macrophage NO and TNFα was not tested in this study. It is also noteworthy that histamine and serotonin are short-lived and their effects are rapidly lost after mast cell degranulation. Heparin is an important constituent of MCG and murine macrophages possess heparin receptors.[30] A previous report has shown that commercial heparin and MCG are capable of inhibiting Fcγ2a receptor mediated phagocytosis in macrophage cell line.[31] Although commercial heparin differs from rat MCG-heparin, it is possible that MCG-heparin may have some modulatory effects on macrophage iNOS and TNFα expression. Our results presented here show that both MCG and MCG-sonicate are capable of degrading TNFα with loss of immunoreactivity and biological activity, and that both secreted and recombinant TNFα are susceptible. This indicates that the decreased levels of TNFα could be partially due to the proteolytic effects of MCG-proteases, in addition to the effect of MCG inhibition of macrophage TNFα mRNA expression. However, this proteolytic effect was only partially reversed by treatment of MCG with a mixture of protease inhibitors. The failure of protease inhibitors to completely abrogate the MCG effect may be due to incomplete inhibition of the protease activity or due to the absence of specific inhibitors for certain proteases.

Mast cell degranulation generates the release of a variety of inflammatory molecules that directly stimulate many cell types including epithelial cells,[32] fibroblasts[33] and endothelial cells.[17] This study is the first evidence that mast cell granules inhibit macrophage TNFα and iNOS mRNA expression. We also found that the mast cell proteases degrade recombinant and macrophage secreted TNFα. The pre- and post-transcriptional regulation of TNFα production in LPS-activated macrophages by MCG may contribute to the inhibition of iNOS mRNA expression.

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