

Gc protein-derived macrophage-activating factor (GcMAF) stimulates cAMP formation in human mononuclear cells and inhibits angiogenesis in chick embryo chorionallantoic membrane assay

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Abstract The effects of Gc protein-derived macrophage-activating factor (GcMAF) have been studied in cancer and other conditions where angiogenesis is deregulated. In this study, we demonstrate for the first time that the mitogenic response of human peripheral blood mononuclear cells (PBMCs) to GcMAF was associated with 3'-5'-cyclic adenosine monophosphate (cAMP) formation. The effect was dose dependent, and maximal stimulation was achieved using 0.1 ng/ml. Heparin inhibited the stimulatory effect of GcMAF on PBMCs. In addition, we demonstrate that GcMAF (1 ng/ml) inhibited prostaglandin E₁- and human breast cancer cell-stimulated angiogenesis in chick embryo chorionallantoic membrane (CAM) assay. Finally, we tested different GcMAF preparations on CAM, and the assay proved to be a reliable, reproducible and inexpensive method to determine the relative potencies of different preparations and their stability; we observed that storage at room temperature for 15 days decreased GcMAF potency by about 50%. These data could prove useful for upcoming clinical trials on GcMAF.

Keywords GcMAF · cAMP · Angiogenesis · Peripheral blood human mononuclear cells

Introduction

GcMAF (Gc-macrophage activating factor) is a potent macrophage-activating factor derived from vitamin D-binding protein, a polymorphic serum glycoprotein with multiple functions also known as a group specific component or Gc protein [1, 2]. In vivo, selective deglycosylation of Gc protein occurs naturally as part of the inflammatory response [3], whereas in vitro, membrane-bound beta-galactosidase and sialidase of activated B- and T-lymphocytes hydrolyze the terminal galactose and sialic acid yielding active GcMAF i.e. Gc protein with N-acetylgalactosamine as the remaining sugar [3].

Besides stimulating macrophages, GcMAF was demonstrated to inhibit tumour-associated angiogenesis in a variety of systems [4–7], and this effect was considered to be associated with GcMAF anti-tumour properties [7]. GcMAF effects on cancer cell-stimulated angiogenesis in a developing organism such as the chick embryo chorionallantoic membrane (CAM), however, have not been studied. In addition, little is known about GcMAF intracellular signalling. There is indirect evidence suggesting interaction of GcMAF with a C-type lectin receptor on the macrophage surface [8], and a preliminary report approved for public release by the U.S. Army Medical Research and Materiel Command described that GcMAF blocked the phosphorylation of a band with the approximate molecular weight of 75 kDa in prostate cancer cell lines [9]. However, we were unable to find any study concerning second messenger formation in GcMAF-treated cells. In order to fill these gaps of knowledge on GcMAF, we studied 3'-5'-cyclic adenosine monophosphate (cAMP) formation in human peripheral blood mononuclear cells (PBMCs) as well as GcMAF effects on cancer cell-stimulated angiogenesis in CAM assay. The rationale for studying cAMP

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among second messengers lays in the observation that increased levels of intracellular cAMP have a negative effect on normal angiogenesis in the CAM assay [10]. In addition, since GcMAF is extracted and purified from mammal blood, and the potencies of different preparations may vary, CAM assay was also chosen because it is a simple and inexpensive method that could be exploited to determine the biological effects of different GcMAF preparations.

Materials and methods

Collection and separation procedure of human peripheral blood mononuclear cells (PBMCs)

PBMCs were collected from healthy volunteers. Whole venous blood (5 ml) treated with EDTA was used within 2 h of drawing. Five ml of anti-coagulated whole blood was carefully layered over 5.0 ml of Polymorphoprep (Axis-Shield PoCAS, Oslo, Norway) and PBMCs harvested according to the manufacturer's instructions.

Preparation of human GcMAF

GcMAF was purified according to the method reported by Yamamoto et al. [11]. Briefly, Gc1F protein was purified using 25-hydroxyvitamin D₃-affinity chromatography [12]. Stepwise incubation of the purified Gc protein with immobilized beta-galactosidase and sialidase yielded GcMAF [13–15]. GcMAF was filtered through a low protein-binding filter, Millex-HV (Millipore Corp., Bedford, MA) for sterilization. Quality control of the preparation of GcMAF was performed for activity, sterility and safety tests. GcMAF was stored at 4°C. This GcMAF preparation was used in all the experiments reported in this study, and when we mention "GcMAF", we refer to this preparation, obtained from Professor N. Yamamoto. In addition, in the experiments reported in Table 5, a commercially available GcMAF preparation, obtained from www.gcmaf.eu, was tested for comparison. This preparation was termed GcMAF-b in Table 5. In other experiments reported in Table 5, GcMAF-b was stored for 15 days at room temperature (25°C) before testing; the compound stored for 15 days at room temperature was termed GcMAF-c.

Cell culture and treatment

PBMCs were seeded and cultured in RPMI 1640 medium containing 2 mM glutamine, 10% fetal calf serum, 100 IU/ml penicillin and 100 mg/ml streptomycin; cells were maintained at 37°C under 5% CO₂ atmosphere.

Treatments were performed in complete culture medium immediately after seeding. PGE₁ and endotoxin (lipopolysaccharide) were purchased from Sigma–Aldrich, Milan, Italy. Heparin (molecular weight, 12.4 kDa; sulphation degree: SO₃⁻/COO⁻, 2.15) was extracted from bovine intestinal mucosa and was obtained from Opocrin, Modena, Italy.

Determination of cell proliferation

Assessment of cell proliferation was determined by Calbiochem Rapid Cell Proliferation Kit (Calbiochem, D.B.A., Milan, Italy) [16]. Each condition was replicated in quadruplicate samples, and each experiment was replicated three times. Differences between experimental points were evaluated by the Student's *t*-test.

cAMP assay

cAMP levels were measured by a competitive EIA assay (Cyclic AMP EIA kit, Cayman Chemical, Ann Arbor, MI, USA). Each condition was replicated in quadruplicate samples, and each experiment was replicated three times. Differences between experimental points were evaluated by the Student's *t*-test.

Chick embryo chorionallantoic membrane (CAM) assay

Fertilized White Leghorn chicken eggs were incubated under routine conditions, and a window was opened in the egg shell at day 3 of incubation (Fig. 1a). Gelatine sponges (1 mm³) were placed on the top of the CAM at day 8. The sponges were then absorbed with 5 ul of compounds to be tested. Sponges containing PBS were used as negative controls; prostaglandin E₁ (PGE₁, 1 mg/ml) and MCF-7 cells (human breast adenocarcinoma, obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia–Romagna, Brescia, Italy) were used as stimulators of angiogenesis as previously described [17]. MCF-7 cells were used at a concentration 50,000 cell/sponge. In each experiment, we used six eggs per experimental point (i.e. six eggs treated with PBS, six eggs treated with PGE₁ and so on). Each experiment was repeated three times. Thus, the reported results refer to a total of 18 eggs per experimental point. Eggs were examined by stereomicroscopy, and positive angiogenesis was considered if new microvessels (in particular, microvessels surrounding the sponge, defined as circumfocal microvessels) had developed. Small (<1 mm diameter), large (>1 mm diameter) and tortuous microvessels were observed at magnification ranging from ×5 to ×10. Angiogenesis was assessed by scoring the circumfocal microvessel number

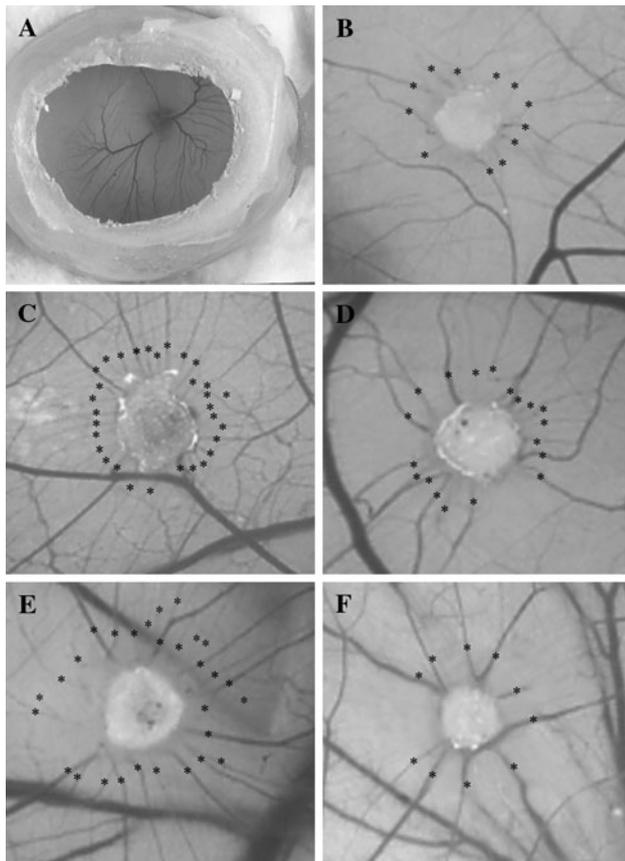


Fig. 1 Evaluation of angiogenesis on CAM assay by stereo-microscopy. **a** Example of a typical window opened in the egg shell at day 3 of incubation. **b** Basal angiogenesis in the presence of PBS or GcMAF (1 ng/ml); only few new blood vessels are present around the sponge. Circumfocal microvessels are indicated with *asterisks*. Circumfocal microvessel number (CFMN) in the picture: 12. **c** PGE₁ (1 mg/ml) stimulates a strong angiogenic response; numerous blood vessels with an irregular course, spiral aspect, and frequent branches surround and infiltrate the sponge. CFMN in the picture: 31. **d** GcMAF (1 ng/ml) significantly inhibits the strong angiogenic response induced by PGE₁ (1 mg/ml); the resulting angiogenesis is weak, very similar to basal angiogenesis depicted in **b**. CFMN in the picture: 19. **e** MCF-7 (cultured human breast cancer cells; 50,000 cell/sponge) induces a significant angiogenic response comparable to that induced by PGE₁ (1 mg/ml). CFMN in the picture: 29. **f** GcMAF (1 ng/ml) inhibits MCF-7-induced angiogenesis; the final angiogenic response is comparable to that observed in **b** CFMN in the picture: 10

(CFMN) [18]. In Fig. 1b–f, circumfocal microvessels are indicated with asterisks. Please notice that the number of visible circumfocal microvessels in Fig. 1b–f is slightly lower than the number easily scored by an observer at the stereo-microscope and reported in Tables 4 and 5. Observers (two for each experiment) were blinded for what concerned the experimental conditions. Differences between experimental points were evaluated by the Student's *t*-test.

Table 1 Human peripheral mononuclear cell proliferation in response to GcMAF

Treatment	Absorbance units at 450 nm
Untreated	423 ± 21
GcMAF 0.01 ng/ml	605 ± 35*
GcMAF 0.1 ng/ml	801 ± 28*
GcMAF 1 ng/ml	798 ± 32*
Lipopolysaccharide	821 ± 50*
Heparin	453 ± 33
GcMAF 0.01 ng/ml + Heparin	425 ± 45
GcMAF 0.1 ng/ml + Heparin	391 ± 47
GcMAF 1 ng/ml + Heparin	477 ± 28

Each compound was added to cell suspension for 24 h at the indicated concentration. Assessment of cell proliferation was determined by Calbiochem Rapid Cell Proliferation Kit (Calbiochem, D.B.A., Milan, Italy), and values are expressed as absorbance units as indicated in (21). The background absorbance is dependent upon the culture medium, pH, incubation time, and time of exposure to light and typical background absorbance was 2–4 absorbance units × 10³. This value was subtracted from all reported measures. Values (absorbance units × 10³) represent means ± SEM (*n* = 12). * *P* < 0.02 versus untreated. Lipopolysaccharide was used at the concentration 1 μg/ml. Heparin was used at the concentration 1 mg/ml

Results

Effects of GcMAF on human PBMCs proliferation

When PBMCs of healthy subjects were challenged with GcMAF for 24 h, we observed a significant response in terms of proliferation (Table 1). The effect was dose dependent, and maximal stimulation was obtained with 0.1 ng/ml GcMAF. The stimulatory effect of GcMAF was comparable to that achieved by the highest concentration of endotoxin (lipopolysaccharide; 1 μg/ml) taken as positive control [19]. Heparin (0.01–1 mg/ml) inhibited the stimulatory effect of GcMAF on PBMCs at all GcMAF concentration tested, presumably by binding the N-acetylglactosamine moiety of GcMAF and/or its receptor (Table 1). In Table 1, we report the results obtained with the highest concentration of heparin (1 mg/ml), even though all heparin concentrations (0.01–1 mg/ml) significantly inhibited GcMAF-induced PBMC proliferation (not shown). The results obtained with the highest concentration of heparin demonstrate that heparin does not inhibit per se peripheral PBMC proliferation. In other words, the results presented in Table 1 rule out the possibility that heparin could have directly inhibited PBMC proliferation, an occurrence that we had previously observed in other cell systems [20, 21]. In addition to the short-term effect at 24 h, GcMAF (0.1 ng/ml) sustained PBMCs proliferation for about 98 h, whereas un-stimulated cells were no longer viable after 48 h (Table 2). Since it is well assessed that

Table 2 Human peripheral mononuclear cell proliferation in response to GcMAF for different time intervals

Time (h)	Absorbance units at 450 nm	
	Untreated	GcMAF 01 ng/ml
24 h	423 ± 21	801 ± 28*
48 h	55 ± 18	958 ± 54*
72 h	17 ± 20	1,264 ± 78*
96 h	18 ± 21	1,015 ± 102*

Typical background absorbance was 2–4 absorbance units $\times 10^3$. This value was subtracted from all reported measures. Values (absorbance units $\times 10^3$) represent means \pm SEM ($n = 12$). * $P < 0.02$ versus untreated

GcMAF does not bind to T- or B-lymphocytes [14], it can be hypothesized that the results obtained challenging PBMCs with GcMAF refer to the effects of GcMAF on monocytes [15]. These results can be interpreted as if, after 98-h treatment, GcMAF had rescued monocytes from apoptosis [22].

Effects of GcMAF on cAMP production

The signal transduction pathway of GcMAF received little attention, in particular as far as intracellular second messengers are concerned; thus, we decided to study cAMP formation in PBMCs. GcMAF (0.1 ng/ml) significantly stimulated cAMP formation in a dose-dependent manner paralleling the results observed by studying cell proliferation (Table 3). The reported increase in cAMP production following GcMAF stimulation was comparable to that previously observed challenging monocytes isolated from PBMCs with 10 nM PGE₂ [23].

Effects of GcMAF on angiogenesis

It was previously demonstrated that GcMAF inhibited angiogenesis and inhibition of angiogenesis was considered a key feature in its anti-tumour properties [4–7]. The effects of GcMAF on angiogenic growth factor-induced cell proliferation, chemotaxis and tube formation were

Table 3 cAMP concentration in human peripheral mononuclear cells in response to GcMAF

Treatment	cAMP
Untreated	226 ± 22
GcMAF 0.01 ng/ml	355 ± 35*
GcMAF 0.1 ng/ml	583 ± 31*
GcMAF 1 ng/ml	568 ± 37*

GcMAF was added for 24 h at the indicated concentration. Values of cAMP concentration (pmol/ml $\times 10^3$) represent means \pm SEM ($n = 12$). * $P < 0.02$ versus untreated

previously examined in vitro by using cultured endothelial cells (murine and porcine cells, human umbilical vein endothelial cells) and in vivo by using a mouse cornea micro-pocket assay. It was demonstrated that GcMAF had direct anti-angiogenic effects on endothelial cells independent of tissue origin. On the basis of these considerations, we studied the effects of GcMAF on the angiogenesis induced by human breast cancer cells in CAM. Thus, we tested its effects both in basal conditions and when administered together with powerful stimulators of angiogenesis i.e. PGE₁ (1 mg/ml), taken as positive control, and the human breast adenocarcinoma cell line, MCF-7. GcMAF did not alter basal angiogenesis, i.e. the angiogenesis observed with PBS (Table 4, Fig. 1b), or chick embryo development (not shown). As expected, MCF-7 cells, directly implanted in CAM, strongly stimulated angiogenesis (Table 4, Fig. 1e) as previously described [17]. Stimulation of angiogenesis by MCF-7 cells was comparable to that induced by PGE₁ (Table 4, Fig. 1c) that is a known inducer of proangiogenic factor production [24]. Figure 1c, e shows that blood vessels with an irregular course and frequent branching were present at day 12; the gelatine sponges were surrounded by allantoic vessels that developed radially towards the implant in a “spoked-wheel” pattern. Tortuous vessels infiltrated the sponges and often modified their shape. GcMAF (1 ng/ml) significantly inhibited PGE₁- and MCF-7-stimulated angiogenesis (Table 4, Fig. 1d, f). It is worth noting that GcMAF concentration required to achieve full inhibition of stimulated angiogenesis was 1 ng/ml, i.e. a concentration tenfold higher than that required to stimulate PBMCs in our experimental conditions. Since CAM assay is a reliable, reproducible and inexpensive method to study angiogenesis, in particular when PGE₁ is used to stimulate angiogenesis, we exploited this method to compare the

Table 4 Quantitative evaluation of angiogenesis on CAM assay

Experimental point	CFMN
PBS	15.3 ± 1.4
GcMAF	16.4 ± 1.8
PGE ₁	35.7 ± 1.2
PGE ₁ + GcMAF	17.4 ± 1.7*
MCF-7	28.5 ± 1.3
MCF-7 + GcMAF	16.7 ± 1.0**

Effects of GcMAF on PGE₁- and MCF-7-stimulated angiogenesis

The average number of blood vessels, expressed as circumfocal microvessel number (CFMN) derived from scoring small (<1 mm dia.), large (>1 mm dia.) and tortuous microvessels, for each experimental conditions. The different compounds were used at the following concentration: GcMAF (1 ng/ml) and PGE₁ (prostaglandin E₁, 1 mg/ml). MCF-7 (cultured human breast cancer cells). Data are reported as means \pm SEM ($n = 18$). * $P < 0.02$ versus PGE₂ (1 mg/ml). ** $P < 0.02$ versus MCF-7

Table 5 Quantitative evaluation of angiogenesis on CAM assay

Experimental point	CFMN
PBS	15.3 ± 1.4
GcMAF	16.4 ± 1.8
GcMAF-b	14.5 ± 1.6
GcMAF-c	14.5 ± 1.6
PGE ₁	35.7 ± 1.2
PGE ₁ + GcMAF	17.4 ± 1.7*
PGE ₁ + GcMAF-b	18.3 ± 1.5*
PGE ₁ + GcMAF-c	26.8 ± 1.5*

Effects of different GcMAF preparations on PGE₁-stimulated angiogenesis

The average number of blood vessels, expressed as circumfocal microvessel number (CFMN) derived from scoring small (<1 mm dia.), large (>1 mm dia.), and tortuous microvessels, for each experimental conditions. The different compounds were used at the following concentration; GcMAF (1 ng/ml), PGE₁ (prostaglandin E₁, 1 mg/ml). Data are reported as means ± SEM (*n* = 18). * *P* < 0.02 versus PGE₂ (1 mg/ml). ** *P* < 0.02 versus MCF-7. GcMAF indicates the compound obtained as described [11]. GcMAF-b indicates the commercially available compound purchased from www.gcmf.eu. GcMAF-c indicates GcMAF-b after 15 day storage at room temperature (25°C)

anti-angiogenic effect of different GcMAF preparations. Table 5 shows that the two GcMAF preparations that we used had about the same potency in inhibiting PGE₁-stimulated angiogenesis. In addition, Table 5 shows that GcMAF storage at room temperature for 15 days decreased the inhibitory effect of GcMAF on PGE₁-stimulated angiogenesis by about 50%.

Discussion

In this study, we demonstrated that GcMAF has potent mitogenic activity on human PBMCs, and stimulation of PBMCs is considered a hallmark of immune system stimulation [25]. Thus, our results are consistent with the reported effects of GcMAF on the immune system [11, 26–31] and with the observation that intramuscular administration of GcMAF (100 ng/week) significantly increased circulating monocyte count in a HIV-positive volunteer after 8 weeks (Pacini and Ruggiero, unpublished results). The observed inhibitory effect of heparin on PBMC proliferation stimulated by GcMAF could be useful to gain insight into GcMAF-receptor interactions. It could also prove useful in upcoming clinical trials, in particular in those trials where GcMAF will be tested as an anti-tumour agent, since heparin is routinely administered to cancer patients who are at increased risk of developing venous thromboembolism [32].

We are aware that the inhibitory effect of GcMAF on angiogenesis might appear counterintuitive. In fact,

tumour-associated macrophages are linked to increase (not decrease) in angiogenesis, and it is well assessed that activated macrophages produce pro-angiogenic cytokines such as interleukin-8, fibroblast growth factor-2 and vascular endothelial growth factor (VEGF) [4]. However, it was previously demonstrated that GcMAF had direct anti-angiogenic effects on endothelial cells in vitro and in vivo and inhibited signalling initiated by different pro-angiogenic growth factors [4]. Therefore, it has been postulated that the anti-angiogenic activity of GcMAF was complementary to the tumoricidal activity of GcMAF-primed macrophages. The direct anti-angiogenic effects of GcMAF were also demonstrated by Kalkunte et al. [6] who reported that GcMAF inhibited human endothelial cell proliferation, blocked VEGF-induced migration, tube formation, and inhibited growth factor-induced microvessel sprouting in rat aortic ring assay. The observed inhibition of PGE₁- and MCF-7-induced angiogenesis by GcMAF on an assay completely different from those used in the previous studies reinforces the hypothesis that the anti-angiogenic effect of GcMAF is independent of the tissue of origin and of the stimulus used to induce the angiogenic response [4]. Inhibition of angiogenesis by GcMAF could then be crucial in determining its effects in conditions where angiogenesis plays a key role in the progression of the disease, from cancer [33] to HIV infection [34].

As far as the stimulatory effect of GcMAF on intracellular cAMP formation is concerned, to our knowledge this is the first time that such an effect is described. GcMAF-induced increase in cAMP formation could also be responsible for its anti-angiogenic effect, since it was demonstrated that elevated cAMP level inhibited angiogenesis in CAM assay [10].

Finally, our results demonstrate that the CAM assay could be proposed as a simple and inexpensive method to determine the relative potencies of different GcMAF preparations and their stability. In fact, according to the literature, the CAM assay has allowed important progress in elucidating the mechanism of action of several angiogenic factors and inhibitors, and the main determinants dictating the choice of this method are its cost, ease of use, reproducibility and reliability [35]. CAM assay allows quantification of interaction of angiogenic substances and anti-angiogenic drugs [35, 36] because blood vessel number can be quantified by a simple morphological evaluation under a stereo-microscope. Our data obtained with different preparations of GcMAF could prove useful for upcoming clinical trials. In fact, it should be noticed that GcMAF is produced by different manufacturers [37–39], and shipping and storage conditions affecting its potency may greatly vary.

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