MYCOLOGY

Effect of granulocyte-macrophage colony-stimulating factor on candidacidal activity of neutrophils, monocytes or monocyte-derived macrophages and synergy with fluconazole

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The effect of in-vitro granulocyte-macrophage colony-stimulating factor (GM-CSF) treatment of neutrophils, monocytes or monocyte-derived macrophages (MDM) on candidacidal activity was tested. Synergy of effector cells with fluconazole (FCZ) for enhanced killing was also investigated. Incubation of neutrophils with GM-CSF 0.67 µg/L plus *Candida albicans* Sh27 for 24 h significantly increased candidacidal activity (36% versus 74%). Synergy with FCZ for killing was also significantly increased from 93% to 97% when neutrophils were incubated with GM-CSF. Monocytes cultured with GM-CSF and *C. albicans* for 24 h had significantly increased fungistatic activity compared to controls and synergy with FCZ for killing was significantly enhanced from 40% to 59%. Monocytes cultured for 3 days with GM-CSF had increased fungistatic activity compared to control MDM and showed synergy with FCZ for significantly enhanced killing (46%) compared to control MDM (12%).

Introduction

The molecular biology and functions of granulocyte-macrophage colony-stimulating factor (GM-CSF) have been described by Metcalf [1]. GM-CSF purified from mitogen-stimulated human T cells was shown to stimulate neutrophil function [2, 3]. GM-CSF produced by recombinant DNA technology has been used clinically in AIDS patients [4, 5]. Treatment of neutrophils or monocytes *in vitro* with rhGM-CSF increased their fungicidal activity for *Candida albicans* [6, 7]; this was associated with enhanced superoxide anion production [8].

A recent study reported that fluconazole, a triazole that is only fungistatic for *C. albicans* showed synergy with human neutrophils, monocytes or monocyte-derived macrophages (MDM) for increased candidacidal activity [9, 10]. Those results helped to explain the curative efficacy of fluconazole *in vivo*. The present study postulates that if GM-CSF-treated neutrophils, monocytes or MDM have augmented fungicidal activity, they should also show synergy with fluconazole for greater candidacidal activity compared to controls.

Materials and methods

Candida albicans

C. albicans isolate Sh27 (ATCC 56882) and clinical isolates 94–159 and 94–186, susceptible to fluconazole (MIC < 0.5 mg/L) [9, 10] were used in these experiments. Yeast cells were grown on blood agar at 35°C for 48 h. Yeast cells were washed in saline, diluted, counted and suspended in RPMI-1640 containing penicillin 100 000 U/L, streptomycin 100 mg/L and fresh frozen human serum (HS) 10% (5% in some experiments) (CTCM). Dilutions of the suspension were plated on blood agar plates in quadruplicate at time zero to determine the number of cfu in the inoculum.

Effector cells

Polymorphonuclear neutrophil leucocytes (PMNL) and peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood by dextran-70 (6%) sedimentation followed by density gradient centrifugation on Histopaque 1077 (Sigma). PMNL were suspended to 2×10^6 /ml in CTCM and dispensed 0.1 ml per microtest plate well (Corning #25860, Corning, NY, USA). PBMC at 5×10^6 /ml of CTCM with or without GM-CSF were dispensed 0.1 ml per microtest well (half area wells, Corning #25870) then

incubated for 2 h at 37°C in CO₂ 5% in air in a CO₂ incubator. After incubation, non-adherent cells were aspirated and the monolayer was washed once with RPMI-1640. In some experiments, quadruplicate sets of adherent cells (monocytes) were incubated in CTCM with or without GM-CSF for 3 days to obtain monocyte-derived macrophages (MDM).

GM-CSF

Recombinant human GM-CSF, Leukine (Sargramostin), was produced and supplied by Immunex Corp., Seattle, WA, USA. GM-CSF (1.5 × 10⁸ IU/mg) was diluted to 7.5 × 10⁵ IU (5 mg/L) in RPMI-1640 and 0.1-ml portions were stored at −80°C.

Fluconazole

Fluconazole (FCZ) was supplied by Pfizer, Groton, CT, USA. FCZ powder was dissolved in distilled water at 2 g/L and stored at 4°C. Desired dilutions were made from the FCZ stock in RPMI-1640.

Effector cell assays

Effector cell cultures were challenged with 0.1 ml of a suspension of yeast cells in CTCM. Some sets of quadruplicate cultures received 0.01 ml of an appropriate dilution of GM-CSF. Other sets of quadruplicate cultures received 0.01 ml of FCZ to give a final concentration of 1 mg/L of both GM-CSF and FCZ. After incubation for 24 h at 37°C in CO₂ 5%, cultures were harvested with distilled water to lyse human cells and release yeast cells. Appropriate dilutions of harvested material were plated on blood agar, colony counts were made after incubation for 48 h at 35°C, and cfu/culture were calculated.

Quantitative analysis

Human serum (10%) in RPMI-1640 inhibits the growth of *C. albicans* by > 90% compared to growth in RPMI-1640 alone [9]. Typical morphology of growth in CTCM was short budding pseudohyphae. In co-cultures with effector cells no extracellular growth of *C. albicans* was evident. When growth in CTCM was harvested, pelleted and examined by microscopy, 92%

of the fungal units (cfu) of strain Sh27 consisted of one to two cells. The average number of cells per fungal unit for strain 94–159 was 1.7 and for strain 94–186 it was 2.47. The number of cells/unit was not different before challenge of cells or after harvest from effector cells lysed with distilled water.

Inhibition of growth, or percentage of fungistasis, was determined by the formula: [1-(experimental cfu/control cfu)] × 100. Candidacidal activity, or percentage reduction of inoculum cfu, was calculated by the formula: [1-(experimental cfu/inoculum cfu)] × 100. Where killing occurred, e.g., experimental cfu ≤ inoculum cfu, fungistasis was defined as 100%. Student's *t* test was used for statistical analysis of data and significance was set at *p* < 0.05.

Results

Effect of GM-CSF on PMNL

Incubation of PMNL with GM-CSF (0.67 or 3.33 µg/L) and *C. albicans* for 24 h resulted in enhanced killing (*p* < 0.01) compared to killing in PMNL cultures without GM-CSF, 74% and 68% *versus* 36% (Table 1). As previously reported, PMNL showed synergy with FCZ for significantly increased killing of *C. albicans* and that was confirmed here, e.g., 36% *versus* 93% killing. Moreover, PMNL in the presence of GM-CSF showed synergy with FCZ to a significantly greater extent for killing than did PMNL plus FCZ, 4.8 × 10² (SD 0.9 × 10²) and 3.4 × 10² (SD 1 × 10²) cfu *versus* 8.1 × 10² (SD 1.3 × 10²) cfu (Table 1).

In a suboptimal system in which CTCM contained only 5% human serum, PMNL failed to kill *C. albicans* in the 24-h assay. The effect of GM-CSF (3.33 µg/L) on PMNL was amplified in this system and killing increased from 0% to 75% (Table 2). Under these suboptimal conditions, PMNL and FCZ showed synergy only for a low level of killing (17%). On the other hand, PMNL with GM-CSF showed synergy with FCZ for a significantly increased level of killing, e.g., 17% *versus* 98% (Table 2).

Table 1. Effect of GM-CSF on neutrophil activity against *C. albicans* Sh27 and synergy with fluconazole

Treatment*	GM-CSF µg/L	FCZ 1 mg/L	Mean (SD) cfu × 10 ³	Killing (%)	p value
Inoculum			11.6 (0.58)		
CFCM 10% HS	0	—	75.0 (5.7)		
PMNL	0	—	7.5 (1.2)	36	**
	0.67	—	3.0 (1.0)	74	<0.01 [‡]
	3.33	—	3.8 (0.90)	68	<0.01 [§]
CTCM 10% HS	0	+	10.4 (1.6)	10	>0.05
PMNL	0	+	0.8 (0.13)	93	<0.01
	0.67	+	0.5 (0.95)	96 [†]	<0.01 <0.01 [‡]
	3.33	+	0.3 (0.10)	97 [†]	<0.01 <0.01 [§]

*PMNL were co-cultured with *C. albicans* in CTCM with or without GM-CSF and with or without fluconazole (FCZ) in quadruplicate 24-h cultures.
[†]Killing by PMNL + FCZ compared to PMNL + GM-CSF + FCZ, *p* < 0.01.
[‡]Show two pairs of comparisons.
[§]Baseline value for comparisons.

Table 2. Effect of GM-CSF on neutrophil activity against *C. albicans* Sh27 and synergy with fluconazole (FCZ) under suboptimal conditions

Treatment*	GM-CSF $\mu\text{g/L}$	FCZ 1 mg/L	Mean (SD) cfu $\times 10^3$	Killing (%)	p value
Inoculum			0.5 (0.1)		
CTCM 5% HS	0	—	28.3 (3.5)		
PMNL	0	—	0.5 (0.1)	0	**
	3.3	—	0.1 (0.03)	75	<0.01 [‡]
CTCM 5% HS	0	+	1.6 (0.2)	0	
PMNL	0	+	0.4 (0.03)	17 [†]	<0.05
	3.3	+	0.01 (0.01)	98 [†]	<0.01 <0.01 [‡]

*PMNL were co-cultured with *C. albicans* in CTCM containing 5% human serum with or without GM-CSF and with or without FCZ in quadruplicate 24-h cultures.

[†]Killing by PMNL + GM-CSF compared to killing by PMNL + GM-CSF + FCZ, $p < 0.01$.

**, [‡]See footnote to Table 1.

When isolates 94–159 and 94–186 were tested, GM-CSF (0.67 $\mu\text{g/L}$)-treated PMNL increased killing from 49% to 62% and from 73% to 84% ($p < 0.01$), respectively. Moreover, GM-CSF-treated PMNL showed synergy with FCZ for significantly enhanced killing of isolate 94–159 from 81% to 85% ($p < 0.05$). Synergic killing of isolate 94–186 by PMNL + FCZ was 96% and this almost complete killing could not be increased by GM-CSF-treated PMNL + FCZ.

Effect of GM-CSF on monocytes

When monocytes were co-cultured with GM-CSF (0.67 $\mu\text{g/L}$) and *C. albicans* for 24 h, growth was inhibited compared to growth in monocyte cultures without GM-CSF ($p < 0.01$) (Table 3). As previously reported, monocytes showed synergy with FCZ for killing *C. albicans* and here killing was 40%; monocytes with GM-CSF and FCZ caused significantly

increased killing, 59% (Table 3). Similar results were obtained in a second experiment.

Effect of GM-CSF on MDM

When monocytes were cultured for 3 days in CTCM containing human serum 10% with or without GM-CSF (0.67 $\mu\text{g/L}$) both types of MDM were highly fungistatic, but not fungicidal for *C. albicans* (Table 4). It can be noted that MDM from GM-CSF cultures caused increased fungistasis, 1.9×10^3 (SD 0.2×10^3) cfu versus 3.8×10^3 (SD 0.3×10^3) cfu. MDM showed synergy with FCZ for killing *C. albicans* (12%). Moreover, synergic killing increased to 46% when GM-CSF MDM were cultured with FCZ and *C. albicans* (Table 4).

When monocytes were cultured under suboptimal conditions, e.g., CTCM containing human serum 5%, MDM from GM-CSF cultures had significantly in-

Table 3. Effect of GM-CSF on monocyte activity against *C. albicans* Sh27 and synergy with fluconazole (FCZ)

Treatment*	GM-CSF $\mu\text{g/L}$	FCZ 1 mg/L	Mean (SD) cfu $\times 10^3$	Killing (%)	p value
Inoculum			1.5 (0.1)		
CTCM 10% HS	0	—	153.0 (50.0)		
Monocytes	0	—	2.2 (0.1)	0	**
	0.67	—	1.5 (0.1)	0	<0.01 [‡]
CTCM 10% HS	0	+	6.4 (1.0)	0	
Monocytes	0	+	0.9 (0.1)	40 [†]	<0.01
	0.67	+	0.6 (0.1)	59 [†]	<0.01 <0.01 [‡]

*Monocytes were co-cultured with *C. albicans* with or without GM-CSF and with or without FCZ in quadruplicate 24-h cultures.

[†]Killing by monocytes + FCZ compared to killing by monocytes + GM-CSF + FCZ, $p < 0.01$.

**, [‡]See footnote to Table 1.

Table 4. Effect of GM-CSF on monocyte-derived macrophages (MDM) against *C. albicans* Sh27 and synergy with fluconazole (FCZ)

Treatment*	GM-CSF $\mu\text{g/L}$	FCZ 1 mg/L	Mean (SD) cfu $\times 10^3$	Killing (%)	p value
Inoculum			1.5 (0.05)		
CTCM 10% HS	0	—	96.7 (10)		
MDM*	0	—	3.7 (0.3)	0	**
	0.67	—	1.8 (0.2)	0	<0.01
CTCM 10% HS	0	+	8.6 (1.7)	0	
MDM	0	+	1.3 (0.09)	12 [†]	<0.01 [‡]
	0.67	+	0.8 (0.16)	46 [†]	<0.01 <0.01 [‡]

*MDM were co-cultured with *C. albicans* in CTCM with or without GM-CSF and with or without FCZ in quadruplicate 24-h cultures.

[†]MDM + FCZ versus FCZ alone, $p < 0.01$, MDM + GM-CSF + FCZ versus MDM + GM-CSF, $p < 0.01$.

**, [‡]See footnote to Table 1.

creased fungistatic activity compared to control MDM, e.g., 1.9×10^3 (SD 0.6×10^3) versus 4.8×10^3 (SD 1.7×10^3) cfu (Table 5). Control MDM failed to show synergy with FCZ for killing *C. albicans* although fungistasis was markedly improved; however, GM-CSF MDM showed synergy with FCZ and, in combination, killed 75% of the yeast cells. (Table 5).

Discussion

This report shows that GM-CSF enhances the anti-fungal activity of PMNL in a 24-h assay. Richardson *et al.* found similar results with a different experimental design [6]; adherent PMNL were pretreated with GM-CSF for 1 h, then challenged for 3 h and enhanced killing of *C. albicans* was measured as reduced survival of the inoculum.

PMNL activated by GM-CSF have increased capacity to show synergy with FCZ, resulting in significantly enhanced killing of *C. albicans* compared to controls.

Sixty percent of *C. albicans* yeast cells are killed by monocytes in 2-h assays [11]. Depending on inoculum size and donor variations it has been found that in 24-h assays there can be reduction of inoculum cfu or, as reported here, fungistasis. We postulate that in 24-h co-cultures, yeast cells surviving the initial killing multiply so that the number of cfu at 24 h is greater than at the time of inoculation.

The present study showed that co-culture of monocytes with GM-CSF for 24 h enhanced their fungistatic activity for *C. albicans*. Smith *et al.* used a different protocol and an indirect method for measuring antifungal activity and reported similar results [7]. They exposed monocytes to GM-CSF (500 U/ml) for 14 h, challenged the monocytes with ³H-leucine-labelled *C. albicans* for 4 h and measured increased antifungal activity by ³H-leucine release. Moreover, the present study demonstrated that GM-CSF induced increased ability of monocytes to show synergy with FCZ for killing *C. albicans* compared to controls.

While others have reported that GM-CSF activation of

MDM increased antimicrobial activity against *Mycobacterium avium*, *Leishmania donovani* and *Trypanosoma cruzi*, this study reports for the first time that GM-CSF increased the fungistatic activity of MDM for *C. albicans* [12–14].

A previous study demonstrated that MDM shows synergy with FCZ for killing *C. albicans* [10]. This study confirms those results and demonstrates that GM-CSF treatment augments the capacity of MDM to show synergy with FCZ for increased candidacidal activity.

These results provide a rationale for combination therapy with GM-CSF and fluconazole in the treatment of fungal infection.

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Table 5. Effect of GM-CSF on monocyte-derived macrophages activity against *C. albicans* Sh27 and synergy with fluconazole (FCZ)

Treatment*	GM-CSF µg/L	FCZ 1 mg/L	Mean (SD) cfu × 10 ³	Killing (%)	p value
Inoculum			1.3 (0.08)		
CTCM 5% HS	0	—	192.5 (3.0)		
MDM	0	—	4.8 (1.7)	0	**
	0.67	—	1.9 (0.06)	0	<0.01
CTCM 5% HS	0	+	5.9 (1.0)	0	
MDM	0	+	1.8 (0.05)	0	<0.01 [‡]
	0.67	+	0.3 (0.06)	75	<0.01 <0.01 [‡]

*MDM were co-cultured with *C. albicans* in CTCM containing 5% human serum with or without GM-CSF and with or without FCZ in quadruplicate 24-h cultures. MDM + FCZ *versus* FCZ, p < 0.01; MDM + GM-CSF + FCZ *versus* FCZ, or *versus* MDM + GM-CSF, p < 0.01. ** , [‡]See footnote to Table 1.

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