Comparison of effects of CH50 on macrophage activation and its anti-tumor activity with those of lipopolysaccharides[1]

ZHANG Gui-Mei, FENG Zuo-Hua[2], LI Dong, ZHANG Hui (*Department of Medical Molecular Biology, Tongji Medical University, Wuhan 430030, China*)

KEY WORDS fibronectins; recombinant fusion proteins; lipopolysaccharides; interferon type Ⅱ; experimental melanoma; macrophage activation

ABSTRACT

AIM: To investigate the characterization of pharmacological action & of CH50, a recombinant polypeptide of human fibronectin, by comparing the effects of CH50 on macrophage activation and its anti-tumor activity with those of lipopolysaccharides (LPS). **METHODS**: The production of nitric oxide (NO) as an index macrophage activation was determined by colorimetric assay. The interferon-γ (IFN-γ) transfection was performed with co-precipitation of calcium phosphate and DNA. The melanoma B16 cells were inoculated into abdominal cavity of mice and the number of tumor nodes was recorded. **RESULTS**: At lower concentrations or when given alone *in vitro*, CH50 produced ten times less NO than LPS ($P < 0.01$). But at concentrations higher than 1 $mg \cdot L^{-1}$, CH50 activated the IFN-γ-primed macrophages to produce NO to the same extent as LPS ($P > 0.05$). There was no synergism between CH50 and LPS. Both CH50 and LPS alone could reduce the number of tumor nodes in abdominal cavity of mice but CH50 had a stronger inhibitory effect on the growth of tumor *in vivo* as compared to LPS ($P < 0.01$). CH50/IFN-γ had also a better inhibitory effect on tumor growth *in vivo* than LPS/IFN-γ did. **CONCLUSION**: In the presence of IFN-γ, the ability of CH50 to activate macrophages is the same as that of LPS. But CH50 has better antitumorogenic effects *in vivo* against mouse melanoma as compared to LPS.

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[2] Correspondence to Prof FENG Zuo-Hua.
Phn 86-27-8369-2636. E-mail myjsz@tjmu.edu.cn

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INTRODUCTION

Polypeptide CH50 is a bifunctional-domain recombinant polypeptide which contains Cell Ⅰ and Hep Ⅱ domains of human fibronectin (Pro1239-Ser1515 connected with Ala1690-Thr1960 through Met)[1,2]. This polypeptide can inhibit the tumor metastasis *in vivo*[2], and enhances the anti-tumorogenic activity of macrophages[3]. We have observed that CH50 and interferon-γ (IFN-γ) have a synergistic effect on the activation of macrophages. It has been known that lipopolysaccharide (LPS) is the most effective factor which acts alone, or together with IFN-γ, to activate macrophages. But LPS can not be used in tumor therapy because of its toxicity *in vivo*. In this study, we investigated the characterization of pharmacological action of CH50 by comparing the effects of CH50 on macrophage activation and its antitumorogenic activity with those of lipopolysaccharides.

MATERIALS AND METHODS

Mice Male Kunming mice (6 – 8 wk, 18 g ± *s* 1 g, Grade Ⅱ, Certificate № 19 – 052) were purchased from Department of Experimental Animals, Tongji Medical University.

Tumor cells Melanoma B16/F1 cells were purchased from ATCC Co, USA. Cells were cultured in RPMI-1640 medium containing 10 % calf serum (Gibco).

Drugs and reagents Recombinant polypeptide CH50 was prepared by Heparin-agarose chromatography (purity 98 %, identified by SDS-PAGE)[1]. LPS was purchased from Sigma Co (St Loius, MO). (lot № 127H4097).

Assay for nitric oxide produced by macrophage Mouse peritoneal macrophages were cultured in 96-well culture plate at a concentration of 2×10^9 $cells \cdot L^{-1}$ for 48 h. The concentration of nitric oxide in supernatant was determined by measuring the stable nitrite end product from NO by colorimetric assay[3].

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***In vivo* transfection of peritoneal cells with interferon-γ gene** Plasmid pIγ7 was constructed by recombination of XhoI-BamHI fragment (full-length cDNA of mouse IFN-γ) with expressing vector pREP8 (Invitrogen, USA). Transfection solution containing coprecipitates of calcium phosphate and DNA was prepared according to the conventional method[4]. And mouse peritoneal cells were transfected with 20 μg DNA ip.

Effect of CH50 and LPS on tumorogenesis The mouse was inoculated by ip injection of 1×10^6 melanoma B16/F1 cells. CH50 was administered at $0.15 - 15$ mg·kg^{-1} ip, once daily for 10 d. As a control, LPS was given ip at $0.15 - 5$ mg·kg^{-1}, once daily for 10 d. When CH50 and LPS were used in combination with IFN-γ in therapy, the same dosage and treatmenttime were used. One day after the therapy, the mice were killed and the number of tumor nodes in peritoneal cavity were recorded according to the size of nodes ($\varphi < 1$ mm or $\varphi \geqslant 1$ mm), which was determined according to the mean value of the length and width of node. The efficacy of therapy was evaluated by the percentage inhibition of the number of nodes. Inhibition % = (number of nodes of control group − number of nodes of treated group) ÷ number of nodes of control group × 100 %.

Statistics The experimental results were analyzed with t test.

RESULTS

Effect of CH50 and LPS on macrophage activation After transfection with IFN-γ gene, the peritoneal macrophages were primed. Both CH50 and LPS activated the primed macrophages in a concentration-dependent manner (Fig 1). At the concentrations lower than 1 mg·L^{-1}, the effect of CH50 declined much faster than that of LPS. The activity of LPS was more than 10-fold higher than that of CH50. But at concentrations higher than 1 mg·L^{-1}, the activity of CH50 was similar to that of LPS. At three concentrations chosen from the plateau range as in Fig 1, the effects of CH50 and LPS had no significant difference on the activation of primed macrophages (Tab 1). But the effects of CH50 and LPS on the activation of unprimed macrophages were significantly different. There was no synergistic effect observed between CH50 and LPS.

When peritoneal macrophages were harvested for 6 h after ip injection of PBS, CH50, or LPS alone, the NO contents in the supernatants of above were 1.2 ± 0.7,

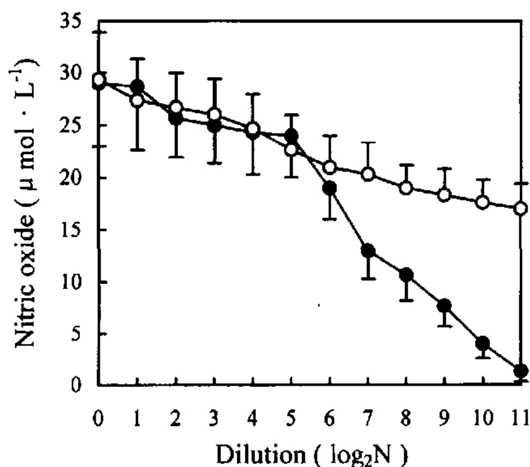

Fig 1. Activation of interferon-γ primed marcophages by CH50 (●) or lipopolysaccharides (○) in a concentration-dependent manner *in vitro*. The initial concentrations ($\log_2 1 = 0$) of CH50 and LPS were 50 and 5 mg·L^{-1}, respectively. N means number of times.

Tab 1. The production of nitric oxide by macrophages stimulated with CH50 and LPS *in vitro*. $n = 8$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* PBS, $^fP < 0.01$ *vs* LPS/0.2, $^iP < 0.01$ *vs* LPS/1.0, $^lP < 0.01$ *vs* LPS/5.0, $^oP < 0.01$ *vs* corresponding CH50 or LPS.

Group/mg·L^{-1}	Nitric oxide (μmol·L^{-1})	
	without IFN-γ	with IFN-γ
PBS	1.3 ± 0.7	3.1 ± 1.2
CH50/2	3.5 ± 3.2^{cf}	24.1 ± 3.1^{co}
CH50/10	5.3 ± 5.1^{ci}	25.3 ± 4.7^{co}
CH50/50	7.3 ± 4.6^{cl}	27.1 ± 5.9^{co}
LPS/0.2	6.3 ± 4.7^{c}	22.6 ± 6.1^{co}
LPS/1.0	10.7 ± 6.4^{c}	25.9 ± 6.5^{co}
LPS/5.0	12.5 ± 6.3^{c}	28.6 ± 8.1^{co}
CH50 + LPS/2 + 0.2	7.1 ± 3.3^{cf}	24.7 ± 6.5^{co}
CH50 + LPS/10 + 1.0	9.8 ± 5.9^{ci}	26.7 ± 6.1^{co}
CH50 + LPS/50 + 5.0	14.1 ± 5.2^{cl}	27.3 ± 7.1^{co}

2.4 ± 1.3, 3.5 ± 1.2 μmol·L^{-1}, respectively ($P > 0.05$ *vs* PBS group). When PBS, CH50, or LPS were injected ip 1 d after IFN-γ-transfection solution, and macrophages were cultured *in vitro* for 48 h, the NO contents in the supernatants were 4.1 ± 1.4, 21.3 ± 2.7, and 17.2 ± 2.1 μmol·L^{-1}, respectively ($P < 0.01$ *vs* PBS group), indicating that the primed macrophages were activated quickly *in vivo* by CH50 and LPS.

Comparison of antitumor effects by ip injection of CH50 or LPS After ip injection of melanoma

Tab 2. Comparison of antitumorogenic effects of CH50 and LPS against mouse melanoma *in vivo*. $n=7$. $\bar{x} \pm s$. [c]$P<0.01$ *vs* PBS, [f]$P<0.01$ *vs* LPS.

Group/mg·kg^{-1}	Tumor nodes ($\varphi<1$ mm)		Tumor nodes ($\varphi\geq1$ mm)	
	without IFN-γ	with IFN-γ	without IFN-γ	with IFN-γ
PBS	137±25	64±16	46±12	15±7
LPS/1.5	74±25[c]	58±17[c]	17±8[c]	7±5[c]
CH50/5	14±7[cf]	22±11[cf]	15±6[c]	2±4[cf]

cells, mice were injected with different dosages of CH50 or LPS alone. CH50 and LPS had the same inhibitory effects upto 1.5 mg·kg^{-1} (Fig 2). At higher doses, the inhibitory effect of LPS declined because of its toxicity (degressive body weight and hair out of order) but CH50 had no such effect.

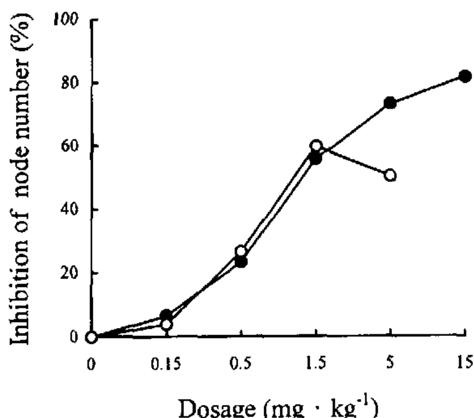

Fig 2. Growth-inhibitory effects of CH50 (●) and LPS (○) alone on mouse melanoma inoculated in abdominal cavity.

On the other hand, the formation of tumor nodes was suppressed by ip injection of CH50, LPS or ip IFN-γ gene transfection. All the three had similar effects on the numbers of tumor nodes bigger than 1 mm but the reduction in the number of tumor nodes smaller than 1 mm was significantly different. CH50 had more potent inhibitory effect (Tab 2). When accompanied by IFN-γ gene transfection, CH50 also produced much stronger inhibitory effects than LPS (Tab 2).

DISCUSSION

VLA-5 is the receptor of Cell Ⅰ domain on the surface of macrophages[5]. The transmembrane signal transduction triggered by either Cell Ⅰ domain or LPS requires the activation of protein kinase C (PKC)[6,7]. The present results indicate that CH50 and LPS activate macrophages *in vitro* in a concentration-dependent manner when they are used alone. Moreover, either CH50 or LPS have a synergistic effect with IFN-γ on the activation of macrophages. But CH50 and LPS themselves have no synergistic effect at different concentrations. The results suggest that CH50 and LPS possess same function in macrophage activation.

There was a threshold observed for the binding of CH50 to its receptor[8] in a recent study from our lab. A sufficient binding capacity was reached at the concentration around 1 mg·L^{-1}. At concentrations lower than this, the binding ability reduced quickly[8]. Similar results were observed when CH50 was used to activate macrophages in this study. At concentrations lower than 1 mg·L^{-1}, the effect of CH50 declined quickly. But LPS acted in a different manner. Its effect lowered gradually along with reduction in its concentration. The results indicate that LPS had a higher affinity for its receptor than does CH50.

A sufficient activation of macrophages needs a double-signalling[4] which can induce macrophage-mediated tumor cytotoxicity most effectively. So far there is no such suitable agent for clinical application in tumor therapy. Although LPS and IFN-γ are most effective for the activation of macrophages, it is difficult to use LPS in clinic therapy because of its toxicity. CH50 was observed to have no toxic effects according to our results and those of Saiki *et al*[9]. Moreover, CH50 can also inhibit the metastasis of tumor *in vivo*[2,8,9]. Thus, CH50 and IFN-γ may be paired as macrophage-activating factors to be used in clinical treatment for tumors. The present results show that LPS has a stronger stimulating effect on macrophages than CH50 does *in vitro*, but CH50 produces a better inhibitory effect on the tumor

growth *in vivo*.

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CH50与脂多糖的巨噬细胞激活作用及抗肿瘤活性的比较[1]

张桂梅, 冯作化[2], 李　东, 张　慧 (同济医科大学医学分子生物学研究室, 武汉 430030, 中国)

关键词 纤连蛋白类; 重组融合蛋白质类; 脂多糖类; 干扰素Ⅱ型; 实验性黑色素瘤; 巨噬细胞活化

目的: 将重组纤连蛋白多肽 CH50 的抗肿瘤活性及巨噬细胞激活作用与脂多糖(LPS)进行比较, 分析 CH50 作为抗肿瘤多肽的药理作用特点. **方法**: 一氧化氮(NO)以比色法进行检测; 采用磷酸钙-DNA 共沉淀法进行 γ-干扰素(IFN-γ)体内基因转染; 腹腔内接种黑色素瘤 B16 细胞并计数肿瘤结节数. **结果**: 体外低浓度或单独作用时, CH50 激活巨噬细胞产生 NO 的作用比 LPS 低 10 倍以上($P<0.01$). 但在体外大于 1 $mg\cdot L^{-1}$浓度且与 IFN-γ 联合应用时, CH50 促进巨噬细胞产生 NO 的作用与 LPS 相同($P>0.05$). CH50 和 LPS 之间没有协同作用. CH50 单用在体内抑制肿瘤生长的作用优于 LPS ($P<0.01$). CH50/IFN-γ 亦比 LPS/IFN-γ 抑制肿瘤生长的作用更强. **结论**: 与 IFN-γ 联合应用时, CH50 对巨噬细胞的激活能力与 LPS 相同; 但是 CH50 在体内具有更强的抑制小鼠黑色素瘤生长的作用.

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