EFFECTS OF GOAT PLACENTAL IMMUNOREGULATORY FACTOR ON NON-SPECFIC IMMUNITY OF MICE

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ABSTRACT

Goat placental immunoregulatory factor (GPIF) is a small molecular weight (MW ≤10,000D) polypeptide extracted after ultrafiltration of healthy puerperal goat placentas. A previous study showed that GPIF had wide biological activities. This study was focused on the nonspecific immunologic enhancement by GPIF on mice. Exposure of Balb/c mice peritoneal macrophages w to 0.05, 0.1, and 0.5 mg/ml GPIF resulted in significant cell proliferation ($p<0.01$), promotion of phagocytic ability ($p<0.05$), and an increase of nitrous oxide (NO) ($p<0.01$) and interleukin-1 (IL-1) ($p<0.05$) secreted by macrophages *in vitro*, in a dose-dependent manner. Five Gy $^{60}Co\gamma$ ray irradiated, immunosuppressed mice were given 25, 12.5, 6.25 mg/kg GPIF respectively by intraperitoneal injection (i.p) for 7 days. This resulted in a a significant increase of phagocytic ability and clearance ability ($p<0.05$). No significant differences were found between 25 mg/kg GPIF treated immuno-suppressed mice and the normal mice *in vivo*, which indicated that GPIF raised the nonspecific immunity of immuno-suppressed mice to normal levels. Our results demonstrated that GPIF might act as an immunological agonist to increase the nonspecific immunity of mice.

Keywords: GPIF, immunologic enhancement, macrophage, nonspecific immunity

INTRODUCTION

Human placental factor (HPF) is a polypeptide compound extracted from healthy puerperal placentas of women (1). HPF has displayed a wide range of biological effects such as recovery of immunosuppressed immunocytes (2); enhanced lymphocyte proliferation (3); and promoted the antibody synthesis (4). Due to the limited resources of human placentas and for ethical reasons, there is an urgent need to seek for novel animal immunoregulatory factor to substitute for HPF (5). Goat placental immunoregulatory factor (GPIF) is a small molecular weight polypeptide extracted from healthy puerperal goat placentas by ultra filtration in our laboratory (6). The previous analysis indicated that its molecular weight ≤10,000; ultraviolet absorption peak 280nm; A_{260}/A_{280} ≥2.0. GPIF displayed extensive immunological activities and Fang reported that GPIF could enhance humoral immunity and recover the ability of humoral immunity in mice with immunodeficiency (7).

GPIF is thus expected to be developed as an immunological drug applied in the clinic to recover the immunological function of tumor patients treated with radiotherapy and chemotherapy. GPIF is classified as a fifth-type novel biological product for treatment according to *Procedures Administration for Drug Registration*. Because its specific immunostimulatory function was described by Gao *et al*.(8), these experiments were designed to investigate the immuno-stimulatory effects of GPIF on nonspecific immunity of mice in accordance with the requirements of an application of a new biological product.

MATERIALS AND METHODS

Animals and reagents

Animal experiments were approved by Committee of Experimental Animal Center, Third Military Medicine University. Balb/c mice (SPF, 6 weeks, 18-22 g, QN 310101014), and an adult rooster (SPF, 1.5 kg) were provided by Experimental Animal Center, Third Military Medicine University. The animals were maintained under standard conditions in an animal house

(10,000-grade sterilized environment) approved by the Committee for the Purpose of Control, and Supervision on Experiments on Animals. The animals were given pelleted foods (Xiwang, Chongqing Ltd., Chongqing, China) and sterilized water ad libitum. GPIF (frozen dry, MW ≤10,000) was prepared in our laboratory and its concentration was estimated by Coomassie Brilliant Blue assay. Calf thymosin (Tα) was purchased from ChongQing SanXin Pharmaceutical Co Ltd. RPMI-1640 was purchased from Gibco (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased form Shanghai Sunway Biotech Co. Ltd. (Sunway, Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), lipopolysaccharide (LPS), concanavalin A (ConA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Neutral red and Giemsa dyes were purchased from ChengDu Kelong Chemical Reagents Factory (Kelong, ChengDu, China). India ink was purchased from BeiJing Xizhong Chemical Reagents Factory (Xizhong, BeiJing, China).

In vitro tests

Preparation of macrophage monolayer

Balb/c mice were injected i.p with 0.5 ml 0.5% starch physiological saline (PS) and sacrificed by cervical dislocation 3 days later, then dipped into 75% ethanol for 30 seconds at room temperature. After the fur was thoroughly dry, the peritoneal cavity was injected with 5 ml D-Hank's solution (pH7.4). The anterior and lateral walls of the abdomen were gently massaged, followed by opening of the peritoneum cavity to collect peritoneum washes on to a Petri dish placed on ice. Washes were filtered thorough a nylon filter (filter pore size 180um, diameter 90mm, Millipore) into a 15 mL Falcon tube placed on ice, pelleted at 1200 rpm, at 4 ^oC for 5 min and resuspended in RPMI-1640 to adjust the cell density to 1×10^6/ml. Cells were seeded in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin (50 IU/mL) and streptomycin (50 IU/mL) in a 96-well plate at 37^oC and 5% CO_2. After the cells had reached 90% confluence, the medium was replaced with fresh RPMI-1640 and the monolayer cells were subjected
to the following assays.

MTT assay

MTT assay described by Mosmann (9) was used to determine peritoneal macrophage proliferation. Peritoneal macrophages monolayer was prepared as above. GPIF was added to a final concentration: 0 (Group A), 0.05(Group B), 0.1(Group C), 0.5 (Group D) mg/mL which were the same as in the following *in vitro* assays. The total medium volume of each well was 200 µl and each dosage was repeated six times in the same plate. After further incubation for 24 h, 20 µl of MTT (5 mg/ml in PBS (pH 7.4, 10 mM)) was added to each well followed by 4 h incubation. The medium was then discarded, 150 µl DMSO was added to each well and incubated for 20 min to dissolve the purple-blue formazan precipitate. The optical density (OD_{570nm}) was measured with microplate reader (550 Bio-Rad, USA) and represented the proliferative stimulation rate.

Phagocytic ability assay

Peritoneal macrophages monolayer was prepared in 96-well plate as above. Fresh RPMI-1640 or same medium containing 0.05, 0.1, 0.5 mg/ml GPIF were added to 96-well plates and incubated for 24 h, followed by discarding the supernatant and adding 100 µl neutral red solution (0.075% PS) for a further 30 min. To each well was added 100 µL lysis-solution (equivalent 50% ethanol plus 50% acetic acid), followed by lysis of macrophages. Due to an existing absorption peak at 570 nm, the OD_{570nm} was measured to indirectly express the phagocytic ability (10).

Nitrous oxide assay Nitrite method was used to determine the amount of nitrous oxide secreted by peritoneal macrophage, and NO content was used to represent the level of nitrous oxide (11). Briefly, the standard curve was first established as follow: $OD=0.0021C+0.0269$ (R^2= 0.9998, X-axis represented the NO content and Y-axis represented OD). Two hundred microliter macrophage suspension (cell density 1×10^6/mL) was added to each well, supplemented with 0.05, 0.1, 0.5 mg/ml GPIF and 10 µg/ml LPS. There was a need to stimulate macrophages to secrete NO for more than 24h, after effective incubation for 48 h, 100 µL supernatant was collected into another plate, followed by addition of 100 µL Griess reagent (equal amounts of 1% *p*-aminobenzene sulfonic acid mixed with 2.5% PBS containing 0.1% ethylenediamine) for a further 10 mins. The OD_{570nm} were measured to calculate the NOontent based on the standard curve.

IL-1 assay

Peritoneal macrophages monolayer was prepared in 24-well plate as above. One ml macrophage suspension (cell density 1×10^6/mL) were added to each well, supplemented with 0.05, 0.1, 0.5 mg/mL GPIF and 10 µg/mL LPS and incubated for 48 h. After that, the cells were harvested, pelleted at 2000 rpm at 4^oC, and the supernatant was collected and stored at -20^oC. Mouse thymocyte proliferation method described by Zhang et al. (12) was used to determine the amount of IL-1 secreted by peritoneal macrophages. Thymus from a 6-weeks old mouse sacrificed by cervical dislocation, was filtered through 100 mesh and 200 mesh in sequence, using sterile technique to adjust the cell density to 1×10^7/ml, and supplemented with 1 µg/ml ConA. One hundred microlilter thymocyte suspension and 100 µl of the above supernatant were similarly added to 96-well plate at the same time and thoroughly mixed. The MTT assay was used to determine the amount of IL-1. Briefly, after

further incubation for 24 h, the optical density (OD_{570nm}) was measured to indirectly represent the amount of IL-1 according to the MTT method.

***In vivo* tests**

Groups design, animal model and administration

Sixty Balb/c mice (SPF, 6 weeks, 18-22 g) were selected at random and divided into six groups (n=10) according to a table of random numbers: high dosage (A, 25 mg/kg GPIF); medial dosage (B, 12.5 mg/kg GPIF); low dosage (C, 6.25 mg/kg GPIF); positive control (D, 10 mg/kg Tα); model group (E, 12.5 mg/kg PS); and normal group (F, 12.5 mg/kg PS). At the beginning of experiment, the mice in groups A, B, C, D, E were irradiated by ^{60}Coγ ray for 5 Gy to establish immunosuppression. Through feeding days 1 to 7, mice in groups A, B, C continuously received 25, 12.5, 6.25 mg/kg GPIF respectively by i.p injection, group D was given 10 mg/kg Tα and group E, F were given 12.5 mg/kg PS.

Phagocytic ability assay

At day 5, each mouse was i.p injected with 0.5 ml 5% starch PS for a further 3 days. On the 8th day, peritoneal cavity was injected with 0.5 ml of 5% chicken red blood cells (CRBC, stored in Alsever's solution at 4 ^{o}C) for phagocytosis during 12 h. Mice were sacrificed by cervical dislocation and peritoneal cavity was injected with 2 mL PS, the abdomen was gently massaged for 1 min, followed by opening the peritoneal cavity to accurately harvest 1 ml peritoneal washes onto a slide and further incubated for 30 min at 37°C in enamel dishes. Cells were fixed by acetone-methanol (1:1) for 5 min and stained with 4% Giemsa-PBS for 30 min. Two hundred cells were counted under an oil immersion lens (Leica DM2500, Germany) to calculate the phagocytosis percentage and phagtocytosis index according to the formula (13) :

Phagocytosis percentage (T) = [(number of macrophages participated in phagocytosis)/200 macrophages]×100%

Phagocytosis index (α) = [(number of CRBC phagocytosed by macrophage)/200 macrophages]×100%

Carbon clearance assay

On day 8, each mouse was injected with 0.05ml/10g India ink through the caudal vein. Withdrawal of 20 µL blood from the orbital veniplex was carried out under ether anaesthesia at 1 min (t_1) and 10 min (t_{10}) respectively. The blood was mixed thoroughly with 2 ml 0.1% sodium carbonate, and the body weight, liver weight, and spleen weight were recorded. The OD_1 (responding to t_1) and OD_{10} (responding to t_{10}) were determined at 680nm, with 0.1% sodium carbonate as the reference solution (14).

Clearance index (K) = lg (OD_1/OD_{10})/($t_{10□}t_1$)

Phagocytosis index (α) = [body weight/(liver weight + spleen weight)]×$K^{1/3}$

Statistical analysis

All data were expressed as mean values ± standard deviation (SD), and analysis of variance (one-way ANOVA) was used for evaluating statistical significance. A value less than 0.05 (P□0.05) and 0.01 (P□0.01) were used for statistical significance. Prior to any analysis, data were tested for normality and variance homoscedasticity.

RESULTS

GPIF promotes the immunological function of normal macrophages

After macrophages were treated with 0.05, 0.1, 0.5 mg/mL GPIF, the cell proliferation, phagocytic ability were determined *in vitro*. Compared to control values, GPIF apparently stimulated cell proliferation ($p<0.01$), and promoted phagocytic ability ($p<0.05$). At the same time, the amounts of inflammatory factor, NO, and pro-inflammatory factor, IL-1, secreted were detected. GPIF significantly increased the amount of NO ($p<0.01$) and IL-1 ($p<0.05$), and the increasing of the GPIF had greater activity. The results showed that exposure of macrophage to GPIF caused a significant activation in a dose-dependent manner (Table 1).

GPIF improved the phagocytic ability of immunosuppressed mice

Normal mice were firstly irradiated by ^{60}Coγ ray of 5 Gy and immunosuppressed experimental animal model was produced. Irradiation in group E significantly decreased the phagocytosis percentage (15.7 ± 1.8%, $p<0.05$) and phagocytosis index (0.22 ± 0.03, $p<0.05$) compared with group F (58.5 ± 4.8%, 0.85 ± 0.09), indicating the success in producing the immunosuppressed mice. The effect of GPIF on phagocytic ability for CRBC by immunosuppressed peritoneal macrophage is shown in Figure 1. Administration of 25, 12 and 6.25 mg/kg GPIF increased the phagocytosis percentage to 55.7 ± 4.7%, 48.0 ± 3.9%, and 32.2 ± 2.2% vs 15.7 ± 1.8% in group E ($p<0.05$), and similarly enhanced the phagocytosis index to 0.89 ± 0.08, 0.64 ± 0.05, 0.46 ± 0.04 vs 0.22 ± 0.03, the control value. At the same time, there was no significant difference among groups A, D and F, which indicated that both GPIF and Tα could raise the phagocytic ability of immunosuppressed macrophages to normal levels. The efficiency of 25 mg/ml GPIF was equivalent to 10 mg/ml Tα.

GPIF improves the clearance ability of immunosuppressed mice

The effects of GPIF on the clearance ability of immunosuppressed mice are shown in Figure 2. The irradiation of group E significantly decreased the clearance index (2.22 ± 0.27%, $p<0.01$) and phagocytosis index (4.15 ± 0.56, $p<0.05$) compared with group F (4.14 ± 0.58%, 5.60 ± 0.71), demonstrating their improved clearance ability. Administration of 25, 12 and 6.25 mg/kg GPIF increased the clearance index to 3.58 ± 0.42%, 3.64 ± 0.48%, and 3.77 ± 0.51% respectively

compared to 2.22 ± 0.27% in group E ($p<0.01$), and similarly improved the phagocytosis index to 5.32 ± 0.64, 5.68 ± 0.68, 5.40 ± 0.62 vs 4.15 ± 0.56 the control value. At same time, there was no significant difference among groups A, B, C, D and F, which indicated that both GPIF and Tα could boost the phagocytic function of immunosuppressed mice to the normal level, and the efficiency of GPIF was equal to Tα.

DISCUSSION

The aim of the present study was to evaluate the nonspecific immunoregulatory effects of GPIF to support the pharmacological data in terms with *Procedures Administration for Drug Registration*. Our results demonstrate that GPIF could significantly promote the proliferation and phagocytosis of macrophages *in vitro* by increasing the inflammatory factor NO and proinflammatory factor IL-1, through releasing an inflammatory factor such as interleukin, IFN (15). Similarly, GPIF further displayed the recovery of nonspecific immunity in immunosuppressed mice *in vivo* as in the previous reports on humoral immunity recovered through increased cell proliferation and secretion of cell factors (8, 16). It can be concluded that GPIF probably activated immunocytes to boost nonspecific immunity to normal levels.

Macrophages are a major cell population of the nonspecific immune system, and play an important role in mounting an inflammatory response by secreting a number of cytokines and chemokines. The *in vitro* tests, macrophages were cultured in the presence of increasing of GPIF from 0.05 to 0.5 mg/mL, resulted in the promotion of cell proliferation and increase of phagocytic ability. Similarly, goat placental peptide increased the white blood cells in dogs (18). Along with the increasing of dosage and prolongation of treated time, GPIF was capable of inducing macrophage growth more rapidly, and macrophage exhibited stronger phagocytic activity in both a dose- and time-dependent manner compared with control macrophages. The reason was probably related to a change of ambient condition after incubation with GPIF, followed by a change of inflammatory factors which might lead to a promotion of cell proliferation and phagocytic ability (12).

Macrophages produce an wide array of chemical substances including enzyme, complement protein, and regulatory factor such IL-1, IL-6, NO, TNF, IFN (19). It was worth mentioning that GPIF was reported to cause the T lymphocyte to release inflammatory factor (8). Activated macrophage could produce a great deal of NO to participate in anti-bacteria and anti-cancer via a series cascade reaction. IL-1 also plays an important role in the inflammatory response of the body (20). The results of increasing of inflammatory factor NO and pro-inflammatory factor IL-1 add further supporting evidence that suggests that GPIF is able to activate macrophages through the release of cytokines.

The reticuloendothelial system (RES) is a diffuse system consisting of phagocytic cells. By determining the decrease in the blood concentration of carbon granules clearance ability and further indirectly reflect nonspecific immunity. There was no significance between GPIF and thymosin, which indicated that both GPIF and Tα possessed the same effect on immunologic enhancement. In addition, the boosting action was dependent on the activation of macrophage.

Base on the results, we concluded that GPIF stimulated macrophages producing inflammatory factor and pro-inflammatory factor such as NO, IL-1, and further might act as an immunological agonist to activate nonspecific immunological function. The data adequately satisfy the acquirement of non-specific immunological function for a novel biological product.

ACKNOWLEDGEMENTS

The authors were especially sincerely grateful to the financial support from National Natural Science foundation of China (No. 30070120).

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Table 1: The effects of GPIF on nonspecific immunity of peritoneal macrophage (n=6 $\bar{x} \pm s$)

Group	GPIF (mg/ml)	MTT (OD)	Neutral Red (OD)	NO_2^- (μmol/L)	IL-1 (OD)
A	0.00	0.299±0.079	0.193±0.017	8.32±0.74	0.345±0.015
B	0.05	0.851±0.116**	0.211±0.022	8.96±1.92*	0.364±0.024*
C	0.10	1.078±0.094**	0.268±0.036*	10.60±2.26**	0.482±0.042*
D	0.50	1.939±0.500**	0.279±0.041*	13.27±2.44**	0.476±0.057*

Means of six determinations ± standard deviation. * P <0.05, * * P<0.01 compared with group A.

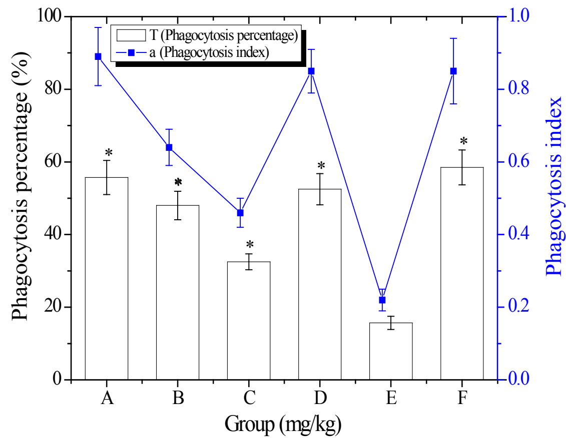

Fig.1.
Phagocytic ability assay (n=10* P <0.05). A: 25 mg/kg GPIF; B: 12.5 mg/kg GPIF; C: 6.25 mg/kg GPIF; D: 10 mg/kg Tα; E: 12.5 mg/kg PS; and F: 12.5 mg/kg PS.

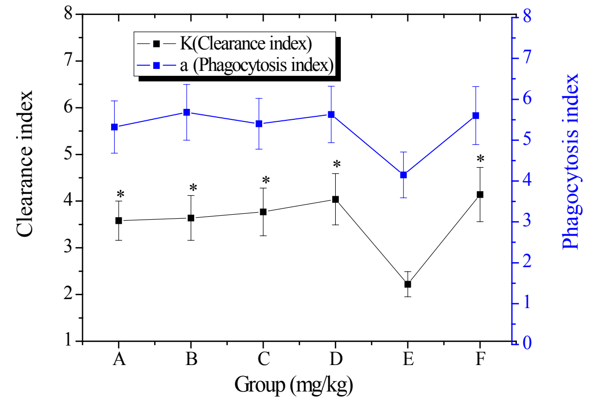

Fig.2. Clearance ability assay (n=10 $\bar{x}\pm s$ * P <0.05). A: 25 mg/kg GPIF; B: 12.5 mg/kg GPIF; C: 6.25 mg/kg GPIF; D: 10 mg/kg Tα; E: 12.5 mg/kg PS; and F: 12.5 mg/kg PS.

Cover image – Lesser Spotted Eagle | *Aquila pomarina*

The Lesser Spotted Eagle (Aquila pomarina) is a common spring and autumn migrant in Israel taking advantage of prevailing thermals for soaring over the region. The autumn passage covers the period, mid-September thro mid-October, and as many as 140,000 eagles have been counted in a single season. The entire world population of A.pomarina apparently migrates over the Middle East.

It is the smallest species of the genus Aquila: body length 60-65 cm, wingspan 130-160 cm, weight 1.0-1.5 kg, and dark brown in colour. It breeds in Russia, Byelorussia, Ukraine, eastern Germany and the Balkans migrating over Israel, central Sinai to over-winter in East Africa, from Sudan to Zimbabwe.