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RESEARCH ARTICLE

Carboxymethytl pachymaram up-regulates dendritic cell function in hepatitis B virus transgenic mice

Anji HOU[1,2], Zhanqiu YANG (✉)[1], Jing HUANG[1], Han JIANG[1]

1 School of Medicine, Wuhan University, Wuhan 430071, China
2 Department of Oncology, Shanghai Seventh People's Hospital, Shanghai 200137, China

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Abstract Carboxymethytl pachymaram (CMP) was administered to HBV transgenic mice through abdominal injection. Lymphocytes were extracted from the spleens. MTT method was used to detect cytotoxicity of CMP. Dendritic cells (DCs) were separated from lymphocytes and incubated with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4). Phenotypes of DC's were assayed by flow cytometry (FCM). IL-12 released by DCs and IL-10 and IFN-γ produced by T cells in mixed lymphocyte reaction (MLR) were measured using ELISA. Results showed that CMP within the concentration of 0–500 μg/mL did not produce cytotoxicity to lymphocytes and could even increase DC phenotypes, and IL-12 level in HBV transgenic mice. It could also increase the secretion of IFN-γ, and inhibit the secretion of IL-10 in MLR. Thus it can up-regulate DC function.

Keywords Carboxymethytl pachymaram, hepatitis B virus transgenic mice, dendritic cell

1 Introduction

It is well accepted that the most important antigen presenting cell (APC) in the human body is the dendritic cell (DC). It plays a key role in the anti-inflammation immune response (Timares L et al., 2003). Studies have demonstrated that DC function in chronic hepatitis B (CHB) patients is suppressed. As a result, the immunological response is inhibited, and the hepatitis B virus (HBV) cannot be cleared out effectively (Kunitani et al., 2002; Pollara et al., 2005). Increasing the immune activity of DCs has been a research hotspot. The method of stimulating DC in vitro and then infusing them into the patients' body or what is called, DC vaccine, has not been applied clinically yet, due to safety considerations (Barratt-Boyes et al., 2000). On the other hand, polysaccharides such as mushroom polysaccharide have been shown to possess good immune regulatory effect and have been used widely in the adjuvant therapy of CHB and malignant tumor (Wang et al., 2006). However, underlying mechanisms of how polysaccharides affect DC function in CHB is still unknown.

Carboxymethytl pachymaram (CMP) is a polysaccharide extracted from *Poria cocos* (Schw.) Wolf. Reviews demonstrate that CMP has anti-tumor and immune regulatory activities. Previously, the experimenters reported that CMP can inhibit secretion of HBsAg and HBeAg in human hepatoma 2.2.15 cell line which is transfected with HBV (Duan et al., 2005). In the present study, HBV transgenic mice served as animal models and treated by abdominal injection of CMP. We investigated CMP's effect on the function of DCs and the differentiation of Th1/Th2 in HBV infected mice.

2 Materials and methods

2.1 Materials and agents

Carboxymethytl pachymaram was provided by Qingyuan Pharmaceuticals from Zhejiang Province, It has the following conditions: purity of 93.3%, molecular weight of 9500, water concentration of 6.5%, arsonium concentration $< 5 \times 10^{-4}$ g/kg, and lead concentration $< 1 \times 10^{-3}$ g/kg. It was then diluted to the needed concentration with D-Hank's solution before use. Cytomycin C and DMSO powder were purchased from Sigma, USA. RPMI1640 medium and fetal calf serum (FCS) were products of GIBCO, USA. Phytohemagglutinin (PHA) was purchased from the Medical College of Peking University. The compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was produced in Duchefa, Holland. Granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-4 (IL-4) were products

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E-mail: yangzhanqiu@163.com

of Cytolab/Peprotech Asia in Israel. FITC-CD80 monoclonal antibody, and PE-CD11c monoclonal antibody were products of BioLegend, USA. PE-MHC-II monoclonal antibody was purchased from eBioscience, USA. IL-12, IL-10,and IFN-γ ELISA kits were products of Biosource, USA. Interferon-α (IFN-α) was a product of Shenzhen Kexing Biotechnology Co. Ltd.

2.2 Animals and treatment

Thirty HBV transgenic mice carrying HBV pre-S, S and X genes, strain: C57BL/6J-TgN (Alb1 HBV)44Bri, male, body weight (20 $\pm$ 2)g, and normal control mice, the same strain, male, body weight (20 $\pm$ 2)g, were purchased from the Experimental Animal Center, Medical College of Peking University. Mice were randomly divided into CMP group, IFN-α group and blank control group, 10 mice in each. In CMP group, mice were treated by abdominal injection of 1% CMP, 0.5 mL/20 g body weight, one injection every three days, for a total of 5 injections. In IFN-α group, α-2b interferon was injected. The dose was 2500 U/20 g body weight at the same interval. In blank control, same volume of normal saline was given to mice at the same interval.

2.3 Separation, purification, and culture of DCs

The experimental procedure according to a previous report (Lunga et al., 2000) was followed. Three days after the last injection, the mice were treated by cervical vertebraectomy and placed in 75% ethanol for 3 min. Spleens were harvested under sterilization, washed 3 times with D-Hank's solution and cut into pieces. These were later filtered through a 200 net nylon mesh. Red blood cells were removed through hypoisotonic method. RPMI1640 medium was added to make monocytes supernate, and then the supernate was placed in 5% CO_2/95% air at 37°C for 3 h. The culture board was shaken slightly. When the supernate was removed, the remaining cells were lymphocytes. These were washed with the RPMI1640 medium culture solution twice. Cells adhering to the tube wall were monocytes, with a small amount of B lymphocytes and T lymphocytes. The cells were counted under a microscope. The same medium culture solution was added to a final cell concentration of 10^6/mL, and then cytokines GM-CSF (2.5×10^{-7} μg/mL) and IL-4 (2.5×10^{-7} μg/mL) were combined. Next, the cells were incubated at 37°C in 5% CO_2/95% air. The culture solution was replaced in half every other day and halves of GM-CSF (1.25×10^{-7} μg/mL) and IL-4 (1.25×10^{-7} μg/mL) were added to it. Matured DCs were harvested after seven days.

2.4 Cytotoxic effect of CMP on lymphocytes

Spleens of HBV transgenic mice were removed, and the monocyte supernates were obtained with the method described previously. The cells were placed in 5% CO_2/95% air at 37°C for 3 h. After centrifugation, the supernates were collected. Cell concentration was regulated to 10^5/mL, and the cells were transferred to 96 well plates, 100 μL per well. 10 μL CMP per well of different concentrations were mixed in. Final concentrations were 4000, 2000, 1000, 500, 250, 125, 62.5, 31.25, 0 μg/mL respectively. Each experiment was done in triplicates. In the control group, CMP was replaced by RPMI1640 medium, and the culture solution was then incubated at an atmosphere of 5% CO_2/95% air at 37°C for 20 h. 10 μL MTT solution was added per well, and incubated for 4 h under the same atmospheric condition. After discard of the supernate, 100 μL DMSO was added per well for 5 min. Value of A was assayed at 570 nm on a full-automatic enzyme analyzer (Immuno-biological Laboratories, INC). Cell damage rate (%) equals (average A value of experimental group $-$ A value of control group)/(A value of cell control group $-$ A value of control group) $\times$ 100%.

2.5 Determination of DCs with flow cytometry

Two milliliters of DCs at 10^6/mL concentration were transferred to a tube, centrifuged at 6500 r/min for 5 min, and washed with pH 7.4 D-Hank's solution twice. Monoclonal antibodies FITC-CD80, PE-CD11c and PE-MHC-II were added respectively, incubated at 4°C for 30 min, and then washed with pH 7.4 D-Hank's solution also. DCs were examined with flow cytometry (Becton Dickinson, USA) and analyzed using the Cell Quest software (Becton Dickinson, USA).

2.6 CMP's effect on IL-2 secretion by DCs

Technique for cell culturing was identical as described previously. Monocyte supernates derived from spleens were incubated at an atmosphere of 5% CO_2/95%, air at 37°C for 3 h. The culture plate was shaken gently. Floating cells were discarded. The culture plate was then rinsed with RPMI1640 medium culture solution twice. Cell concentration was regulated to 10^6/ml with the same solution. GM-CSF (2.5×10^{-7} μg/mL) and IL-4 (2.5×10^{-7} μg/mL) were added, and then IFN-α (final concentration 1 000 U/mL) and CMP (final concentration 125 μg/mL) were added respectively. In control group, CMP was replaced by RPMI1640 medium culture solution and the incubation condition was the same as mentioned above. The culture solution was replaced in half every other day and half doses of cytokines(1.25×10^{-7} μg/mL) were added until day 7. After centrifugation, the supernates were stored at −20°C for assay of IL-12.

2.7 Mixed lymphocyte reaction (MLR)

Monocytes were derived from the spleens of HBV transgenic and normal mice using the method mentioned

above. According to a previous report (Dakic et al., 2004), PBS solution was used to remove platelets. Monocytes were cultured in a tube wall adhering state for 1 h. Floating cells were collected as homologous T cells, whose survival rate was more than 95% with taipan blue staining. The cell concentration was modulated to 1 × 10^6/mL. DCs were collected and the concentration was regulated to 1 × 10^5/mL with PRMI1640 medium solution. Cytomycin C was added (final concentration 25 μg/mL). The DCs were incubated at 37°C for 45 min. A hundred μL DCs per well was blended to the culture plates, and 100 μL homologous T cells derived from the spleens of normal mice was added per well (cell concentration 1 × 10^5/mL). The two kinds of cells were mixed at a ratio of 1:1. Subsequently, 10 μL PHA + CMP, CMP or PHA was added (final concentrations of CMP and PHA were 125 μg/mL, and 10 μg/mL respectively). In control group, RPMI 1640 medium solution was put in and cultured continuously for six days. After culturing finished, the cells were precipitated, and the supernates were collected and stored at −20°C for the IL-10 and IFN-γ testing. The remaining cells were added with MTT solution (5 mg/mL) 10 μL per well and cultured in 5% CO_2/95% air at 37°C for another 5 h. Then, 100 μL acidulated isopropanol was added per well. This was shaken for 5 min and assayed at 550 nm on a full-auto enzyme analyzer.

2.8 Assay of IL-12, IL-10 and IFN-γ

The ABC-ELISA method was used. Each step was strictly performed according to the instruction in the kit.

2.9 Statistics

The program used for statistical analysis was the Statistical Package for the Social Sciences version 11.5 for Windows. Data were expressed as means ± SD. Analysis used was ANOVA, and the level of significance set at $p < 0.05$.

3 Experimental results and discussion

3.1 Cytotoxic effects of CMP on lymphocytes in mice

Results of the MTT assay showed the final concentrations of CMP to be 4000, 2000, 1000, 500, 250, 125, 62.5, 31.25, and 0 mg/mL, respectively. Rates of cell damage were (31.8 ± 0.4)%, (67.5 ± 8.3)%, (70.0 ± 1.6)%, (90.9 ± 1.2)%, (94.4 ± 0.6)%, (153.5 ± 13.1)%, (74.5 ± 6.2)%, (77.5 ± 3.0)%, and (75.4 ± 0.8)%, respectively. Cytotoxic experiment revealed that CMP at the concentration of 0–500 μg/ml did not show cytotoxic effect on lymphocytes of transgenic mice. The best protective concentration on lymphocytes was 125 μg/mL. The 95% confidence interval was 0–2000 mg/L, which suggested CMP's safety on the lymphocytes of HBV transgenic mice.

3.2 Flow cytometry results of DCs of transgenic mice

The results of FCM are shown in a scattergram which were divided into four quadrants according to the X and Y axis and represented by upper left (UL), upper right (UR), lower left (LL), and lower right (LR), respectively (Figures 1 and 2). Area UL represents the positive cell of X axis, LR corresponds to the positive cell of Y axis, LL is the double negative cell, and UR, the double positive cell. The abscissa signifies the height of CD80 marked by FITC. The ordinate indicates the height of CD11c and MHC-II marked by PE. Apparently, the expression of CD80$^+$CD11c$^+$ or CD80$^+$ MHC-II$^+$ were low in HBV transgenic mice. Table 1 also shows the FCM results of DCs.

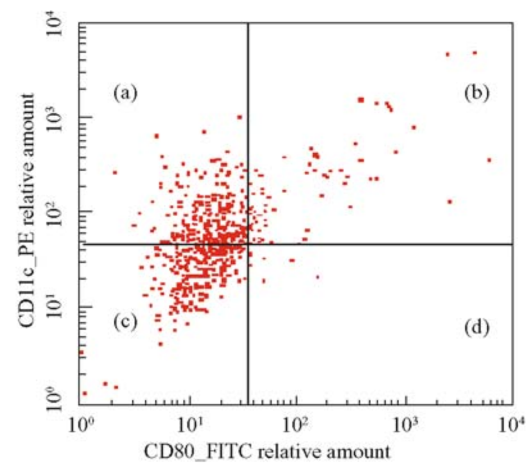

Fig. 1 Results of CD80, and CD11c in HBV transgenic mice by FCM. Four quadrants (a, b, c, d) are UL, UR, LL, LR. Ratios of gated cells are 41.17%, 9.60%, 48.54%, and 0.70% respectively

Double-marked fluorescent antibody technique (FAB t) could be used to measure two different cytokines, provided that these two were marked by different fluorocromes, i.e., CD80 and CD11c as two different cytokines, when observed under a cryptoscope, can be stimulated and observed twice in the same visual field, and photographed. Then the photographs from the two observations are overlapped. Thus the sub-location of antigen A and B in a cell can be studied. Cytokines CD80-FITC, CD11c-PE, and MHC-II-PE have to be divided into two groups for observation: CD80-FITC, CD11c-PE as one group, CD80-FITC, MHC-II-PE as another group. The single positive and double positive cells in each group can be assayed; double positive cells are more likely to be regarded as DCs. As shown in Table 2, CD80$^+$, CD11c$^+$, CD80$^+$ CD11c$^+$ and CD80$^+$, MHC-II$^+$, CD80$^+$ MHC-II$^+$ were the relative amount of single or double positive cells marked by PE and

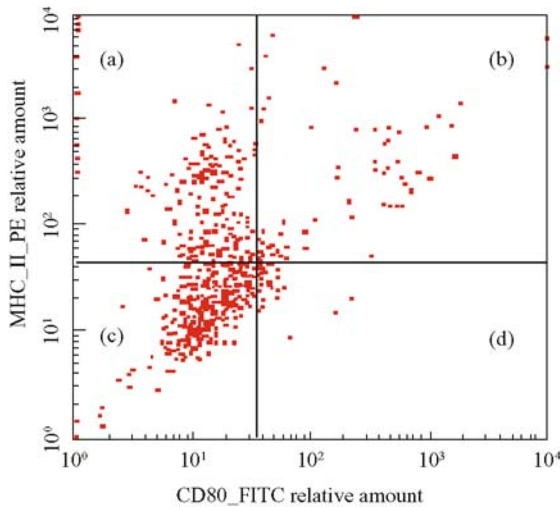

Fig. 2 Results of CD80, and MHC-II in HBV transgenic mice by FCM. Four quadrants (a, b, c, d) are UL, UR, LL, and LR. Ratios of gated cells are 26.95%, 9.80%, 58.65%, and 4.61% respectively

Table 1 Effects of CMP on CD80, CD11c, MHC-II$^+$ of HBV transgenic mice by FCM(%)

DC phenotypes	CMP group	IFN group	control group
CD80$^+$	$32.56 \pm 10.39^{**}$	$33.03 \pm 11.31^{**}$	10.3 ± 4.62
CD11c$^+$	$72.19 \pm 21.81^{**}$	$74.42 \pm 24.09^{**}$	50.77 ± 16.22
CD80$^+$CD11c$^+$	$20.90 \pm 10.66^{**}$	$23.48 \pm 12.00^{**}$	9.60 ± 4.53
CD80$^+$	$24.22 \pm 11.95^{**}$	$29.41 \pm 15.03^{**}$	14.41 ± 5.78
MHC-II$^+$	$44.91 \pm 24.13^{**}$	$47.83 \pm 24.38^{**}$	36.75 ± 11.55
CD80$^+$ MHC-II$^+$	$24.70 \pm 12.17^{**}$	$23.61 \pm 11.98^{**}$	9.80 ± 5.72

Note: compared with control,$^{**}P<0.01$

FITC and assayed by FCM. As shown in Table 1, CD80$^+$, CD11c$^+$, CD80$^+$CD11c$^+$ or CD80$^+$, MHC-II$^+$, CD80$^+$ MHC-II$^+$ were the results of single and double positive cells marked by PE and FITC. It was found out that each phenotype in blank control was lower when compared with other groups, suggesting lower expression of DC phenotypes in HBV transgenic mice. In CMP and IFN groups, expression of DC phenotypes was higher than blank control. When compared between CMP group and control group or compared between IFN and control group, the differences were very significant ($P<0.01$). However, when compared between CMP group and IFN group, the difference was not significant ($P>0.05$).

Determination of DCs included phenotypes of cell and the different cytokines (or receptors of cytokines). At present, there are about a dozen cytokines or cytokine receptors used. However, the dyes used by FCM are only a few. The fluorescent double mark method is commonly employed.

Sub-groups of Th1 and Th2 are derived from CD4$^+$ T lymphocytes. The Th1 type cytokines include IL-2, IL-12, IL-18, IFN-γ and TNF-β. These can stimulate natural killer cell (NK), cytotoxic T lymphocytes (CTL) and macrophages, as well as CTL and immune response in localized inflammation. They can also play an important role in the anti-HBV response. Cytokines with Th2 type include IL-4, IL-5, IL-6, IL-10 and IL-13. Their main function is to promote proliferation of B cell, activation of eosinophils and production of antibodies in humoral immunity. The Th1 and Th2 cytokines from CD4$^+$ T lymphocytes co-regulate the immune response of the human body. These two kinds of cells control each other, and maintain dynamic balance. In some diseases, the antigen continually presents itself and cannot be cleared easily. The Th0 cells have a remarkable tendency to differentiate into Th1 or Th2 cells (MacDonald ET AL., 2002). DCs can activate Th0 strongly and show a super-high efficiency of antigen presentation. It is now widely accepted that the deficient function of DCs causes the defective CTL reaction in CHB infection in the human body (Goncalves ET AL., 2004). The ability of migration to the T cell area after capturing HBV by immatured DCs in CHB patients decreases (Beckebaum et al., 2003). CD40 is expressed at a low level, and the activation of T cells is blocked (Ebner et al., 2001). The co-stimulating factors of CD80, CD86, and CD1a on DC surface and molecules of MHC-II such as HLA-DR are expressed at low levels too (Wakkach et al., 2003). Their ability to stimulate T lymphocyte proliferation *in vitro* decreases, while the level of IL-12 in DC culture supernates declines. Results revealed that the function of DCs in HBV transgenic mice was low, which further supported the conclusions drawn by other researchers. CMP and IFN could up-regulate the low expressed phenotypes of DCs.

3.3 Determination of cytokine level

Levels of IL-12 and IFN-γ in CMP group were markedly higher than control group respectively($P<0.05$); while

Table 2 levels of cytokines ($\bar{x} \pm s$, ng/L)

groups	number	DC supernate IL-12	MLR supernate	
			IL-10	IFN-γ
CMP group	10	$191.00 \pm 61.54^*$	$47.23 \pm 12.40^*$	$101.67 \pm 22.59^*$
IFN group	10	$213.33 \pm 63.28^*$	$48.90 \pm 13.35^*$	$59.00 \pm 18.08^*$
control group	10	48.62 ± 23.87	74.77 ± 16.86	25.33 ± 6.51

Note: *: Compared with Control, $P<0.05$

there was no significant difference between CMP group and IFN-α group($P > 0.05$). IL-10 level in CMP group was noticeably lower than control group ($P < 0.05$), whereas the differences between CMP and IFN-α group were not significant($P > 0.05$)(Fig. 3). It indicates that both CMP and IFN-α can enhance secretion of IL-12 and IFN-γ, and inhibit secretion of IL-10. Outcome of IL-12 in DC supernate, IFN-γ and IL-10 in MLR supernate are shown in Table 2, from which we could draw the same conclusion equally from Fig. 3.

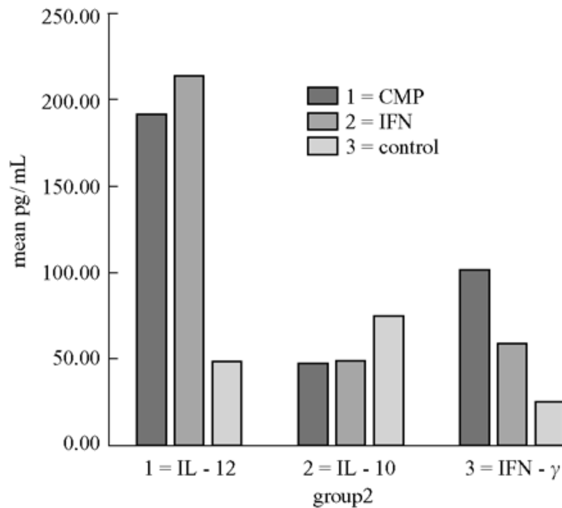

Fig. 3 Levels of cytokines in the supernates of DCs and MLR

3.4 Correlations among cytokines

IL-12 and IFN-γ have a significant positive correlation ($P < 0.05$); however, the correlation between IL-12 and IL-10 or between IL-10 and IFN-γ was not significant ($P > 0.05$) (Table 3).

Table 3 Correlation analysis among cytokines in HBV transgenic mice

cytokines	*r*	*P*
IL-12/IL-10	−0.6490	0.082
IL-12/IFN-γ	0.9113	0.002
IL-10/IFN-γ	0.4113	0.311

r: coefficient

The immune regulatory effect of CMP has been confirmed for years. Yang et al. (1998) reported that CMP alone, or with PHA/Con A can stimulate human peripheral blood lymphocytes (HPBL) to secrete IL-2, IL-6, and IFN-γ at high levels. Chen (Chen, 2002) reported that CMP can apparently enhance phagocytosis of macrophagocytes in peritonium of tumor-loading mice, and remarkably promote secretion of TNF-α by macrophages in the abdomen of mice. In the present study, CMP's effect on the secretion of IL-12 produced by DCs derived from the spleen, and the secretion of IL-10 and IFN-γ produced in the reaction of MLR of HBV transgenic mice was investigated. The suppressing effect of CMP on DC function was observed. The results revealed that because of the low expression of CD80, the co-stimulators on DC surface and MHC-II type molecules, the ability of DC stimulating T lymphocytes to proliferate decreased. IL-12 level in DC culture supernate and IFN-γ in the supernate of MLR decreased. On the other hand, IL-10 level increased and Th1/Th2 cytokines lost their balance. As a result, HBV's exist persistently in CHB patients.

In conclusion, CMP can facilitate secretion of IL-12 through DC generation from the spleen and enhance secretion of IFN-γ in MLR, and inhibit secretion of IL-10. IL-12 and IFN-γ were positively related, suggesting that CMP can regulate the balance of Th1/Th2 cytokines and upregulate DC function. This may be one of the mechanisms of immune regulating effect of polysaccharides.

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