Influence of granulocyte macrophage colony stimulating factor and tumor necrosis factor on anti-hepatoma activities of human dendritic cells

Jin Kun Zhang¹, Jin Lun Sun², Hai Bin Chen¹, Yang Zeng¹ and Yao Jun Qu¹

Subject headings: dendritic cells; granulocyte-macrophage colony-stimulating factor; tumor necrosis factor; anti-hepatoma cell activities; in vitro; peripheral blood

Zhang JK, Sun JL, Chen HB, Zeng Y, Qu YJ. Influence of granulocyte-macrophage colony stimulating factor and tumor necrosis factor on anti-hepatoma activities of human dendritic cells. World J Gastroenterol, 2000;6(5):718-720

INTRODUCTION
Dendritic cells (DCs) play a key regulatory role in antitumor immunity, especially in its immune accessory role via MHC-I molecules[1-5]. We have recently reported that DCs were able to enhance the killing activity of Lymphokine and PHA activated killer (LPAK) cells in vitro[6-8]. In the present study, we evaluated the effects of GM-CSF and TNF upon antitumor activities of freshly isolated dendritic Cells in human peripheral blood (DC-0) and those cells cultivated for 36 hours in vitro (DC-36). To perform such an evaluation, we compared killing effects of LPAK cells with additional DC-0 or DC-36 on hepatoma cell line (BEL-7402) under regulation of GM-CSF or TNF. This study provided some basic data for further antitumor research.

MATERIALS AND METHODS

Tumor cell line
Human hepatoma cell line BEL-7402 was purchased from experimental center of Sun Yat-Sen University of Medical Sciences.

Isolation of DCs
According to our previous method[9], peripheral blood mononuclear cells (PBMC) from healthy volunteers were prepared by using Ficoll-Hypaque (ρ = 1.077g/L) centrifugation method. Interface cells were collected and washed three times to remove platelets. Discontinuous Percoll density gradient centrifugation (Percol: Pharmacia, Sweden) was employed, and then interface cells between 35% and 50% were collected which were called as preliminary enrichment of DCs and divided into two shares. One share (DC-0) was panned immediately; other one (DC-36) further cultured in PRMI-1640 with 100mL/L inactivated fetal calf serum (100mL/L FCS PRMI-1640) at 37°C in a full humidified 50mL/L CO₂ atmosphere for 36 hours, and panned. The non-adherent fractions of two shares as DC-0 and DC-36 were washed and collected for the experiments.

Preparation of LPAK cells[9]
The PBMCs were prepared in the same procedure as above, cultured 2×10⁹/L-popolulation with the final concentration of rhIL-2 1000ku/L and PHA 20mg/L in 100mL/L FCS PRMI-1640 at 37°C in a full humidified 50mL/L CO₂ atmosphere for 7 days. Half volume of the solution was replaced by fresh culture medium at the fourth day.

Anti-tumor experiment
The anti-tumor experiments were divided into two groups and each contained five experimental subgroups. Two ratios of effect (LPAK) to target (BEL-7402) (5:1 and 10:1) were used in all groups. ¹ DC-0 group: d group: BEL-7402 (8×10⁷/L) + LPAK + DC-0 (8×10⁹/L); g1 group: d group + GM-CSF (500 ku/L); g2 group: d group + GM-CSF (100 ku/L); t1 group: d group + TNF (5000 ku/L); t2 group: d group + TNF (500 ku/L); ² DC-36 group: each experimental group was the same as that in DC-0 group except DC-36 in place of DC-0 in the same concentration. These experimental groups were called D group, G1 group, G2 group, T1 group and T2 group respectively. In addition, L groups as the corresponding control groups, BEL-7402 + LPAK, experimental control group only consisted of BEL-7402, its population was 8×10⁹/L. Culture medium control group only contained 100mL/L FCS-PRMI-1640 with the supernatant of LPAK cells at a concentration of 50µL/culture well. All of these
groups were cultured in 96-well-culture plates and each group had 3 wells at 37°C in a full humidified 50mL/L CO₂ atmosphere for 48 hours. Cytotoxicity assay was detected by using neural red uptake method.

**Cytotoxicity assay (neural red uptake method)**

0.1mL 0.3mL/L neural red solution was added in each well for another 1 hour of culture. Following three washings with phosphate-buffered saline (PBS), 0.1mL HCl-ethanol solution was added in each well. The absorption value (A value) of each well was immediately read by BIO-RAD 3550-UV type automatic ELISA reader at 570nm wavelength. The formula of cytotoxicity is as follows:

\[(1 - \frac{\text{Experimental group A - medium control group A}}{\text{Control group A - medium control group A}}) \times 100\%\]

The experimental results were analyzed through analysis of variance by using GB-STAT statistic software. The experiment repeated four times at the same condition.

**RESULTS**

### Influence of DC-0 and DC-36 on cytotoxicity activity of LPAK cells

The cytotoxic activity of L group, d group and D group was enhanced when their ratios of effect to target increased \((P<0.01)\). Their cytotoxic activity were D group > d group > L group \((P<0.01)\) respectively while in the same ratio of effect to target (Figure 1).

### Influence of GM-CSF on DC-0 and DC-36 in helping LPAK cells killing effect

When there were two ratios of effect to target, cytotoxic activity of g1 group and g2 group were obviously higher than d group \((P<0.01)\), meantime, cytotoxic activity of G1 group and G2 group were greatly higher than D group \((P<0.01)\). However, the difference between g1 group and g2 group was not distinct \((P>0.05)\), also there were no difference between G1 group and G2 group \((P>0.05)\). But there were obviously different between g1 group and G1 group \((P<0.01)\), at the same time, the difference between g2 group and G2 group were distinct \((P<0.01)\) (Figure 2).

### Influence of TNF on DC-0 and DC-36 in helping LPAK cells killing effect

While there were two ratios of effect to target, cytotoxic activity of t1 group or t2 group was evidently higher than that of d group \((P<0.01)\), meantime, cytotoxic activity of T1 group or T2 group was markedly higher than that of D group \((P<0.01)\). However, the differences between t1 and t2 group, and between T1 and T2 group were distinct \((P<0.01)\). Furthermore, there were difference between t1 and T1 group \((P<0.01)\), at the same time, between t2 and T2 group \((P<0.01)\) (Figure 3).

**DISCUSSION**

In recent years, it is considered that mature DCs in human peripheral blood have high stimulating function, which efficiently presents tumor-peptide epitopes leading to induce cytotoxic T lymphocytes (CTL) to produce stronger specific antitumor immune response\(^{[11-14]}\). LPAK cells after 7-day induction chiefly express similar phenotype with the CD₁₆⁺, CD₈⁺, CD₃⁺ CTL subtype\(^{[15-18]}\). In our experiments, cytotoxic activity in D group was obviously higher than in d group, which demonstrated that the proportion of mature DCs in DC-36 group was higher than those in DC-0 group and DC-36 group could stimulate LPAK cells to exert stronger antitumor immune response. This effect suggested that a lot of precursor cells of DCs and immature DCs in freshly isolated DCs could...
differentiate into mature DCs after 36h cultivation, which coincided with Young’s opinion[19]. In human peripheral blood, however, not all the precursor cells and immature DCs are able to automatically differentiate into mature DCs in vitro. It is GM-CSF that promotes the differentiation, maturation and activation of DCs. GM-CSF can not only promote the differentiation, maturation and activation of DCs, but also upregulated CD86 expression on DCs, which make DCs to have activating and controlling antitumor immune function[20-24]. Cytotoxic activity difference between G2 (g2) group and G1 group was longer than that in DC-0 group, not all factors from one factor but from combination of multiple factors. In addition, the different time required in different developing stage of DCs populations must be considered. Although developing time in DC-36 group was longer than that in DC-0 group, not all DCs in DC-36 group differentiated into mature DCs. Therefore, increasing GM-CSF concentration alone was senseless. This phenomenon may be helpful in further study of antitumor immunity and clinical research.

In the case of TNF addition, cytotoxic activity was increased greatly, this finding attributed to three roles of TNF: 1, TNF is able to serve as the first signal, which affects DCs development in their early stage or whole stage, leading to upregulate the GM-CSF receptor level of DCs[23]. The supernatant of LPAK cells with minor quantity of cell growth factors such as TNF was added into culture medium to afford synergetic effect with GM-CSF; 2, TNF upregulates expression of CD80, CD83, CD86 and MHC-II in a short period[25,26]. Because these molecules are crucial for efficient antigen presenting, they promote the differentiation, development and activation of DCs. 3, TNF itself can kill tumor cells directly[27-32]. Compared with two t groups, cytotoxic activity of T1 and t1-1 groups were higher than that of T2 and t2 groups, which showed that in DC-36 groups there was plenty of time for immature DCs to evolve into mature DCs after the addition of TNF. Furthermore, T1 (t1) group had higher cytotoxic activity than T2 (t2) group, which was further increased when TNF dosage was raised. This phenomenon may attribute to antitumor effect of TNF itself and the synergetic effect between LPAK cells and TNF.

In conclusion, as compared with uncultured DC-0, cultured DC-36 from freshly isolated DCs had greater cooperative effect with GM-CSF or TNF. Moreover, they enable DCs to fulfill stronger antitumor effect.

REFERENCES

1. Girolomoni G, Ricciardi-Castagnoli P. Dendritic cells hold promise for immunotherapy. Immunol Today, 1997;18:102-104
2. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature, 1998;392:245-252
3. Austyn JM. Dendritic cells. Curd Opin Hematol, 1998;5:3-15
4. Liu SC, Yuan SZ. Relationship between infiltration of dendritic cells, pericancerous lymphocytic reaction and prognosis in colorectal carcinomas. Xing Xiaoxuezhi Yingshi Zhongbao, 1995;3:56-57
5. Hu JY, Wang S, Zhu JG, Zhuo GH, Sun QB. Expression of B7 costimulation molecules by colorectal cancer cells reduces tumorigenicity and induces anti tumor immunity. World J Gastroenterol, 1999;5:147-151
6. Zhang JK, Chen HB, Sun JL, Zhuo YQ. Effect of dendritic cells on LPAK cells induced at different times in killing hepatoma cells. Shijie Huanren Xuebao, 1999;7:673-675
7. Sun JL, Zhang JK, Chen HB, Cheng JD, Qiu YQ. Promoting effects of dendritic cells on LPAK cells killing human hepatoma cells. Zhongguo Zhongliu Yu Kangfu Xuebao, 1998;5:16-18
8. Chen HB, Zhang JK, Huang ZL, Sun JL, Zhuo YQ. Effects of cytokines on dendritic cells against human hepatoma cell line. Shijie Huanren Xuebao, 1999;7:191-193
9. Zhang JK, Chen HB, Wang J, Sun JL. Separating method of dendritic cells. Jiepop Kexue Jinchao, 1998;4:272-273
10. Zhu MS, Yuan AL, Zhang WD, Liu SD, Lu AM, Zhou DY. Dendritic cells in vitro induce efficient and special anti tumor immune response. Shijie Huanren Xuebao, 1999;7:161-163
11. Li MS, Yuan AL, Zhang WD, Chen XQ, Tian XH, Piao YJ. Immune response induced by dendritic cells induce apoptosis and inhibit proliferation of tumor cells. Shijie Huanren Xuebao, 1999;7:56-58
12. Xiao LF, Luo LQ, Zou Y, Huang SL. Study of the phenotype of PBLs activated by CD28/CD80 and CD2/CD58 and acting with hepatoma cells. Shijie Huanren Xuebao, 1999;7:1044-1046
13. Liu SC, Yuan SZ. Relationship between infiltration of dendritic cells and the restricted usage of TCR Vα and Vγ. J Exp Med, 1997;18:616-625
14. Morrison MA, Zhou LJ, Tedder TF, Lyerly HK, Smith C. Generation of dendritic cells from peripheral blood monocytes. Curr Opin Hematol, 1999;6:292-295
15. Zou QY, Li RB, Zheng PL, Chen YZ, Kong XP. Effect of embryo extracts on proliferation and differentiation of hepatoma BEL 7402 cells. Shijie Huanren Xuebao, 1999;7:243-245
16. Borre MA, Zhou L, Ljubow FK, Smith C. Generation of dendritic cells in vitro from peripheral blood mononuclear cells with granulocyte macrophage colony stimulating factor induced dendritic cell development. Blood, 1998;32:3019-3028
17. Huaren Xiaohua Zazhi, 1996;8:1013-1016
18. Chen Q, Ye YB, Chen Z. Activation of killer cells with soluble gastric cancer antigen combined with anti CD-3 McAb. World J Gastroenterol, 1999;5:179-180
19. Zhang JK, Sun JL, Chen HB, Zhuo YQ. Ultrastructural comparison of cells induced at different times in killing hepatoma cells. Shijie Huanren Xuebao, 1998;6:877-879
20. Huaren Xiaohua Zazhi, 1999;7:1044-1046
21. Young JW, Steinman RM. The hematopoietic development of dendritic cells: a distinct pathway for myeloid differentiation. Stem Cells, 1996;14:376-387
22. Stirling B, Gestreasser PR. Dendritic cells: a major story unfolds. Immunol Today, 1995;16:330-333
23. Santiago Schwarz F, Divaris N, Kay C, Carsons SE. Mechanisms of tumor necrosis factor granulocyte macrophage colony stimulating factor induced dendritic cell development. Blood, 1998;32:3019-3028
24. Cao X, Zhang W, Wang J, Zhang M, Huang X, Hanada H, Chen W. Therapy of established tumor with dendritic cells using granulocyte macrophage colony stimulating factor genetically modified dendritic cells. Immunology, 1999;97:616-625
25. Chiodoni C, Paglia F, Sinigüaro A, Rodolfo M, Pannella M, Colombo MP. Dendritic cells infiltrating tumors cotransduced with granulocyte/macrophage colony stimulating factor (GM-CSF) and CD40 ligand genes take up and present endogenous tumor associated antigens, and prime naïve mice for a cytotoxic T lymphocyte response. J Exp Med, 1999;189:125-133
26. Zou YQ, Li RB, Zheng PL, Chen YZ, Kong XP. Effect of embryo extracts on proliferation and differentiation of hepatoma BEL 7402 cells. Shijie Huanren Xuebao, 1999;7:243-245
27. Morss MA, Zhou LJ, Ljubow FK, Smith C. Generation of dendritic cells in vitro from peripheral blood mononuclear cells with granulocyte macrophage colony stimulating factor, interleukin-4, and tumor necrosis factor-α for use in cancer immunotherapy. Ann Surg, 1997;226:6-16
28. Austyn JM. New insights into the mobilization and phagocytic activity of dendritic cells. J Exp Med, 1996;183:1287-1292
29. Zhang W, Huang ZY, Deng YQ, Zhang WD. Lovo cell line apoptosis induced by cycloheximide combined with TNFα. Shijie Huanren Xuebao, 1999;7:326-328
30. Yang SG, Yang ZC, Huang YS, Liu ZY, Fu QF, He BB, Li A. Effect of delayed rapid fluid resuscitation on liver function in early stage postburn. World J Gastroenterol, 1997;3:965-969
31. Wang JY, Wang XL, Liu P. Detection of serum TNF-α, IL-6 and IL-8 in patients with hepatitis B. World J Gastroenterol, 1999;5:38-40
32. Wu MC. Progress in surgical treatment of primary hepatocellular carcinoma. Shijie Huanren Xuebao, 1999;7:921-927