In vitro study of immunosuppressive effect of apoptotic cells*

ZHANG Wen-jin (张文瑾), ZHENG Shu-sen (郑树森)*

(Department of Hepatobiliary and Pancreatic Surgery, First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310027, China)

*E-mail: zhengss@mail.hz.zj.cn

Received Aug. 9, 2004; revision accepted Nov. 19, 2004

Abstract: Recent studies revealed that apoptotic cells are actively involved in immunosuppression and anti-inflammation. After being phagocytosed by macrophages, apoptotic cells can actively regulate cytokines secretion from lipopolysaccharide (LPS)-stimulated macrophages, in which the secretion of immunosuppressive cytokines such as interleukin-10 (IL-10) is increased while the pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNFα), interleukin-1beta (IL-1β) and leukin-8 (IL-8) are suppressed. In this paper, we first present evidence that phagocytosed apoptotic cells regulate cytokine secretion of LPS-stimulated macrophages, but also inhibit the activation of T lymphocytes stimulated by ConA. These data suggest that apoptotic cells can alter the biological behavior of macrophages which gain immunosuppressive property.

Key words: Macrophages, Apoptosis, Cytokines, Immunosuppression

INTRODUCTION

Apoptosis plays an important role in differentiation, development and pathophysiological processes such as inflammation, neoplasia and autoimmune diseases. For a long time, apoptotic cells per se and the clearance of apoptotic cells had been viewed as neutral in immune response. Recently many investigations suggested that apoptotic cells actively regulate the immune response (Voll et al., 1997; Fadok et al., 1998; 2000; Byrne and Reen, 2002). Apoptotic cells release latent and also active TGFβ1, a potent immunosuppressant, which contributes to the establishment of local immunosuppressive milieu (Chen et al., 2001). Reiter and Krammer (1999) found that necrotic tumor cells help macrophages kill tumor cells, but that, apoptotic tumor cells decrease the competence of macrophage in clearance of tumor cells, and enhance prompt the growth of tumor cells. In addition, phagocytosis of apoptotic cells was found to inhibit macrophage activity and finally stimulate the growth of Trypanosome cruzi (Frierie-de-Lima et al., 2000; Freitas Balanco et al., 2001). These findings indicate that apoptotic cells can induce immune tolerance. Necrotic cells send a "danger signal" to the immune system and thus evoke strong immune response, while cells dying by apoptosis do not send a danger signal to the immune system, and finally induce T lymphocytes to become tolerant. However, if apoptotic cells are not engulfed effectively, they will undergo secondary lysis, which could send out danger signal to the immune system and finally result in immune response (Savill, 1998; Savill et al., 2002). This causes immunologists to give second thought to the significance and clearance of apoptotic cells. Savill et al.(2002) suggested that the phagocytosis of apoptotic cells should not only be viewed as clearing aging cells to make room for functional cells, but also setting up the immunosuppressive milieu at the local site. Interestingly, some investigations revealed that after apoptotic cells were bounded or ingested by macrophages, the secretion of immunosuppressive cytokines such as IL-10 was increased markedly, whereas the secretion of
pro-inflammatory cytokines such as TNFα, IL-12 and IL-1β were suppressed (Voll et al., 1997; Fadok et al., 2000; Byrne and Reen, 2002). These results imply that apoptotic cells or phagocytosis of apoptotic cells may actively regulate immune response. Here we also found that apoptotic cells, after being ingested by macrophages, can actively alter the biological behavior of macrophages, which acquire immunosuppressive property.

MATERIALS AND METHODS

Antibodies and reagents

RPMI-1640 and hank balanced salt solution (HBSS) were purchased from GIBCO (America). Lipopolysaccharide (LPS), canavalin A (ConA), FITC-Annexin-V and propidium iodide (PI) were purchased from BD Pharmingen (America). The monoclonal antibodies hamaster anti-mouse FITC-CD69 (Cat# HM4001), rat anti-mouse TRITC-CD3 (Cat# RM3406), rat anti-mouse PE-CD25 (Cat# RM6004) and Isotype control antibodies hamaster IgG-FITC, rat IgG2a-TRI (Cat# R2a06), rat IgG1-PE (Cat# R104), rat Ig2a-PE (Cat# R2a04) were obtained from CALTAG (America). Monoclonal anti-TGFβ1 antibody (Cat# MAB2401) was from RD. Mouse TGFβ1, TNFα and MIP-2 ELISA sets were from American Biosource Company.

Cell culture and treatment

Human T lymphocyte Jurkat cells were purchased from Academy of Medical Science, China. They were cultured in RPMI-1640 containing 10% (V/V) heat-inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% (V/V) CO2. Macrophages were prepared by peritoneal lavage from abdominal cavity ICR mice. Briefly, spleen cells were harvested and cultured in RPMI-1640 containing 10% heat-inactivated FCS for 20 h before stimulation by ConA. Jurkat T cells were used as the source of apoptotic cells. When the density of Jurkat T cells reached 5×10^6 ml^-1, the cells were irradiated by ultraviolet (254 nm) light for 10 min (Fadok et al., 1998) and then incubated for 2 h to obtain 60%–80% apoptosis cells, while necrosis cells were less than 5% (assessed by flow cytometry). To obtain necrotic (lysed) cells, Jurkat cells were irradiated by ultraviolet (254 nm) light for 20 min. The average percentage of lysis, assessed by trypan blue positive, was approximately 90%.

Female ICR mice (6–8 weeks old, 20–25 g) were obtained from Animal Experimental Center of Zhejiang University. The mice were stimulated with LPS (0.5 μg/g) intraperitoneally. After 3 d, the mice were sacrificed by CO2 asphyxiation, and their peritoneal cavities were lavaged with HBSS. Cells were pelleted at 300 g (10 min) and resuspended in RPMI-1640. The cells were then plated at 4×10^6 cells per well in six-well tissue culture plates and allowed to adhere for 2 h. Nonadherent cells were washed off and the remaining macrophages were cultured in RPMI-1640 without serum. Apoptotic Jurkat cells were added at a ratio of five per macrophage, and then cultured for 24 h. Necrotic Jurkat cells or viable Jurkat cells were added to macrophages as control. Supernatants were harvested 24 h later.

For our experiment, the cells were divided into four group: Apoptotic group: apoptotic Jurkat cells were added to macrophages; Necrotic group: necrotic Jurkat cells were added to macrophages; Viable group: viable Jurkat cells were added to macrophages as a control group; Anti-TGFβ1 group: anti-TGFβ1 neutralizing antibody was added to macrophages together with apoptotic Jurkat cells.

Ratio of apoptotic cells induced by UV irradiation

After UV (254 nm) irradiation, the cells were resuspended in 100 μl HBSS, followed by the addition of 10 μl FITC-Annexin-V and 10 μl PI. The cells were then incubated for 15 min in the dark at room temperature, washed with HBSS, and then finally analyzed on an FACSC alibur flow cytometer. The ratio of apoptotic cells was calculated by the following equation:

\[
\text{Ratio of apoptotic cells} = \frac{\text{Number of FITC-Annexin-V positive and PI negative cells}}{\text{Total number of cells irradiated by ultraviolet light}}
\]

Ratio of phagocytosis of apoptotic cells

In order to assess the percentage of phagocytosis of apoptotic cells, 5×10^6 previously labelled apoptotic Jurkat cells were added to each well of macrophages (the ratio of apoptotic Jurkat cells to